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DIAGNOSTIC ADAPTATION OF TIME-RESOLVED FLUORIMETRY IN A MULTI-USE DNA DETECTION ASSAY

ERIKA MIREILLE PFEIFFER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Virology.

DEPARTMENT OF MEDICAL MICROBIOLOGY
AND INFECTIOUS DISEASES

Edmonton, Alberta

FALL, 1994

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DEGREE: Master of Science in Virology

YEAR THIS DEGREE GRANTED: 1994

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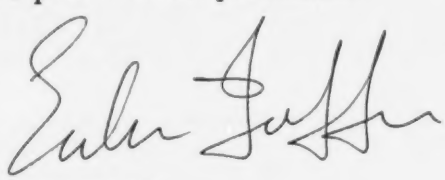

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So, please hold my hand
As we blunder through the maze
And remember
Nothing can grow without rain.

Roger Waters, The Pros and Cons of Hitch Hiking, 1984.

Words are a heavy thing..... they weigh you down. If birds talked, they couldn't fly.

Marilyn Whirlwind, Northern Exposure, "On Your Own"

Good or bad, curiosity is woven into our DNA like tonsils or like the opposable thumb. It's the fire under the ass of human experience!

Chris Stevens, Northern Exposure "The Final Frontier"

To discover and to teach are distinct functions; they are also distinct gifts, and are not commonly found united in the same person.

John Henry, Cardinal Newman. *On the Scope and Nature of University Education;* preface

Ethics and science need to shake hands.

Richard Clarke Cabot. *The Meaning of Right and Wrong;* Introduction

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DIAGNOSTIC ADAPTATION OF TIME-RESOLVED FLUORIMETRY IN A MULTI-USE DNA DETECTION ASSAY submitted by ERIKA MIREILLE PFEIFFER in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN VIROLOGY.

Raymond G. Marusyk

William L. Albritton

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Laurence Jewell

August 22, 1994

To: Barbara Gail Hamilton-Pfeiffer

a scientist, a role-model, my mom.

ABSTRACT

DNA hybridization assays are gaining acceptance in clinical and research laboratories. These assays offer absolute specificity and high sensitivity. Of the detection methods available, those using radioactive labels are the most commonly used because of their sensitivity. However, radiolabels pose health hazards and management difficulties. New non-radioactive labels have been developed with the hopes of satisfactory sensitivity without the hazards of radioactivity. Polymerase chain reaction (PCR) is commonly used to amplify DNA to levels detectable by agarose gel electrophoresis and ethidium bromide staining. A generally unrecognized disadvantage of PCR is its extreme sensitivity: contamination of aerosolized or carried–over DNA is difficult to overcome without specialized equipment and careful manipulations.

One newly investigated DNA hybridization detection system uses time-resolved fluorimetry (TRF). The main advantage of TRF is the ability to discriminate background and diagnostic fluorescence resulting in high sensitivity. The main label used in TRF is europium. Some studies have combined PCR with time-resolved fluorimetry. Both these techniques are sensitive enough to be used independently. This research was designed to demonstrate that TRF is accessible to laboratories without PCR equipment. The assay was designed around a flexible solid support mechanism such that it could be used to detect any specific DNA sequence sought. The support mechanism developed in this study captures a biotin-labelled probe to the target

DNA and the captured DNA is then detected by an europium-labelled probe.

Labelling techniques include labelled phosphoramidites, aminolink, transamination, tailing of labelled nucleotides with terminal transferase, ligation of unlabelled and labelled segments with T4 RNA ligase, unidirectional PCR, and double stranded probe unlabelled strand deletion.

Of the many labelling methods investigated, the europium– and biotin–labelled probes found to be most adaptable to routine use in clinical laboratory were easily constructed and specific. The levels of sensitivity of the probes tested of this assay were theoretically 4 fmol (40 pg) of DNA, and, using 50 ng/ml of europium–labelled probes, 25 fmol to 1 pmol of HAd3 DNA. This project makes this technology accessible to many clinical and research laboratories. It also gives these laboratories the power to expand their investigation of a particular DNA sequence without having to rely upon rigid and expensive kits.

ACKNOWLEDGMENTS

To incorporate some of my personality into this work, I have included a dedication and a selection of quotations which I found characterized my time in the Department of Medical Microbiology and Infectious Diseases, and how I currently look at research and the world in general. Now, I would like to record in print, for all eternity, my thanks to the many people without whom I never would have accomplished my personal and educational goals.

I would like to start by expressing my thanks to Dr. Ray Marusyk, my supervisor, for first taking me on as a graduate student and for providing direction and advice regarding my research, as well, I want to thank him for allowing me the creative freedom to pursue my own research ideas and to fulfill my own goals. I want to thank Dr. William Albritton for serving as a member on my committee, and for providing the back-up support services of Research and Development. Also in R. & D., I would like to thank Linda Chui for all the times I needed technical and trouble-shooting advice. As well, I thank Linda for her friendship. Also, I would like to thank Dr. Glen Armstrong, for advice and for serving as a committee member, and Dr. Laurence Jewell for serving as my external committee member. I thank all my committee for their kind patience.

Special thanks goes to Andrew Chau for personal and technical advice and for being there when I needed him (always). I also want to thank my mother, Barbara G. Hamilton-Pfeiffer, and my best friend, Dr. Kathy A. Nicol for their unbounded support and confidence.

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LIST OF ABBREVIATIONS

Å	- ångstrom
amol	- attomole
APS	- ammonium persulfate
ATP	- adenosine triphosphate
BSA	- bovine serum albumen
°C	- degrees Celsius
cm	- centimetres
CS	- calf serum
dCTP	- deoxycytosine triphosphate
dUTP	- deoxyuracil triphosphate
DMSO	- dimethylsulfoxide
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleotide triphosphate
ELISA	- enzyme-linked immunosorbent assay
EtOH	- ethanol
Eu	- europium
EIA	- enzyme immunoassay
EDTA	- ethylenediamine tetracetic acid
FBS	- fetal bovine serum
fg	- femtogram
FIA	- fluoroimmunoassay
fmol	- femtomole
ft^2	- square feet
g	- gram
G	- gauge
HAd3	- human adenovirus 3
HEp-2	- human epithelial cell line 2
HPLC	- high performance liquid chromatography
HRP	- horse radish peroxidase
h	- hour
Inc.	- incorporated
M	- molar
mAmps	- milliamperes

MEM	- minimal essential medium
min	- minutes
mg	- milligrams
ml	- milliliter
mM	- millimolar
mm	- millimetres
MOI	- multiplicity of infection
OD	- optical density
OPC	- oligo purification cartridge
OPD	- o-phenylenediamine
PBS	- phosphate buffered saline
PCR	- polymerase chain reaction
%	- percent
PFU	- plaque forming units
pg	- picogram
pmol	- picomole
PNK	- polynucleotide kinase
psi	- pounds per square inch
®	- registered
RNA	- ribonucleic acid
RPM	- revolutions per minute
SA	- streptavidin
sec	- second
Sm	- samarium
taq	- *Thermus aquaticus*
Tb	- terbium
TBE	- TRIS borate EDTA
TE	- TRIS EDTA
TEMED	- N,N,N',N'-tetramethylethylenediamine
™	- trademark
Tm	- melting temperature/annealing temperature
TRF	- time-resolved fluorometry
TR-FIA	- time-resolved fluoroimmunoassay
TRIS	- TRIS (hydroxymethyl) aminomethane
TPBS	- TRIS phosphate buffered saline
tween	- polyoxyethylenesorbitan

U	- unit
µg	- microgram
µl	- microlitre
µM	- micro molar
µs	- microsecond
UV	- ultraviolet
V	- volt
v/v	- volume per volume
w/v	- weight per volume
X	- times
Xg	- times the force of gravity

INTRODUCTION

A. Rationale

In the field of diagnostic medicine, researchers and clinicians find themselves in a never–ending battle to identify and fight pathogenic microorganisms. Once a microbial disease is discovered, the causative microorganism must be characterised. The researcher develops tests that specifically detect the particular pathogen. The clinical microbiologist applies these standard tests to isolate and identify the intruder. A test must be precise in its identification, as certainty is essential. However, the organism has the ability to randomly mutate and change its character and develop resistance against treatments prescribed by the physician. As a result, these tests become obsolete and the researcher must develop a new way to identify the pathogen, and the physician, a new way to treat it.

The clinical microbiologist has a battery of tests available (morphologic, biochemical, immunological, and genetic) and each has been designed to be precise in their diagnosis; however, with this precision is the inability to compensate for changes in the pathogen. There may be variances in the appearance, biochemistry, antigenicity, and DNA sequences between strains; therefore, what is required is a test that is accurate but still flexible enough to cope with changes in a particular organism.

Tests traditionally available to the clinical microbiologist are often unsuitable. Immunoassays in general, although sensitive, are limited to the specificity of the particular antibody used. Antibodies are

produced in live animals and are thus influenced by the host–organism's immune response; as well, the immune response generated depends on the antigen used. A standard antibody preparation may not be suitable due to cross–reactivity with other components within a individual sample. Strains within microbial species may vary slightly with regards to antigenicity and, in this instance, the antibody is designed to detect an entire class of microorganisms; in this case, cross–reactivity is desirable. In addition to the variables associated with antibody–based assays, there are ethical considerations regarding the use of animals in research and medicine; when an equal or superior alternative is presented, the option to use animals should be excluded.

There are many different labelling systems used in immunoassays and DNA hybridization assays. Many have unfavourable characteristics which make them less than ideal. Fluorescent labels, such as fluorescein and rhodamine, are partly quenched by immunoreaction. When used as direct DNA labels, they are ineffective due to low sensitivity from background interference (Urdea *et al.*, 1988). Other fluorescent probes, such as dansyl chloride, may suffer due to interference from the serum, which fluoresces at a wavelength similar to the labels. As a result, when choosing a label, a wavelength exceeding 500 nm and a large Stoke's shift is desirable so as to avoid this interference due to serum in the sample (Soini & Hemmilä, 1979). Colourimetric detection systems, used with either immuno– or DNA hybridization assays, are often restricted by background interference from innate sources, because of incomplete removal of unbound label, or from the colour of the samples

themselves. Radioactive labelling systems (^{35}S, ^{32}P, ^{125}I) although sensitive, are less desirable to work with as they pose a serious health hazard if not handled and disposed of correctly. In addition, many have short half–lives (14 days for ^{32}P and 87 days for ^{35}S), adding to expense and making materials management awkward for a clinical or research laboratory. Because of their extreme sensitivity, imprecise use may easily raise the frequency of false positives. Although sensitivity is a goal in the development of a diagnostic procedure, highly sensitive systems have the potential to complicate the interpretation of results. If a system is too sensitive, it will recognize microorganisms at levels below which could cause disease. No system is perfect in all respects; the type of assay and the label chosen depends upon the desired parameters of the test.

Time–resolved fluorimetry is relatively new, but has become an established technique in the expanding area of DNA hybridization methodology. With the advances made in recombinant DNA technology, nucleic acid hybridization systems have become important tools in basic research as well as in the clinical laboratory for the detection of infectious, neoplastic, and genetic diseases. The foundation work of many researchers has shown that time–resolved fluorimetry, when applied to DNA hybridization assays, has value in clinical diagnostics (Dahlén, 1987, Dahlén *et al.*, 1987, Dahlén, *et al.*, 1988, Dahlén *et al.*, 1991a, Dahlén *et al.*, 1991b, Hurskainen *et al.*, 1991).

One method which is used to increase the sensitivity of a particular DNA hybridization assay is to incorporate polymerase chain reaction (PCR). Although simple in design, this is a powerful tool to amplify sections of undetectable amounts of DNA (theoretically, one

copy) to levels which are easily demonstrated by less sensitive detection systems. However, PCR itself is susceptible to contamination by DNA carry–over between samples, and, because it can amplify minuscule amounts to detectable levels, the chance of false positive results are high (Persing, 1991). There are means to prevent this: extremely careful manipulations using positive–displacement pipettes or aerosol–barrier tips, irradiation of stock solutions with UV light, or incorporation of dUTP, rather than dTTP, into the product followed by treatment with uracil–N–glycosylase prior to subsequent manipulations will all help to reduce the potential of contamination. Nevertheless, these precautions are time–consuming and difficult in the clinical laboratory. In the expanding field of time–resolved fluorimetry, PCR has been an almost absolute component; however, because of the problems associated with PCR, my research has avoided using this technique in the actual assay.

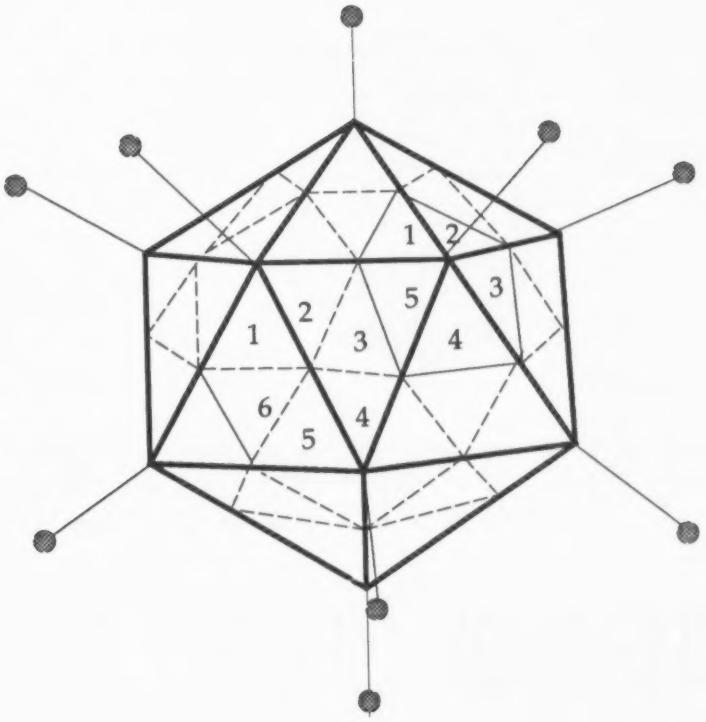
My research goal was to fulfill this diagnostic niche; to develop an assay that is highly specific, sensitive, and flexible. It has been developed with the specificity of DNA hybridization and the sensitivity of time–resolved fluorimetry, but using a specific capture mechanism (Hurskainen *et al.*, 1991) which allows the technique to be applied with any probe. Rather than using completely different tests or methods, one test may be applied to many samples simultaneously, saving time and money.

B. Human adenovirus type three

Human adenovirus type three (HAd3) belongs to the family of viruses known as Adenoviridae. These viruses possess a regular icosahedron–shaped capsid with 20 triangular sides. In addition, they also possess 12 spike–like structures, called fibres, which project from each of the icosahedron's 12 vertices. The capsid is made of protein and is composed of two distinct and basic structures, penton bases and hexons, totaling 252 capsomere subunits. The penton base and hexon capsomeres are formed by the arrangement of different polypeptides, five and six, respectively, to each. The manner in which these polypeptides interact with one another determines the penton base or hexon structure; the pattern of arrangement of these penton and hexon capsomeres with one another forms the icosahedron (Figure 1). In addition to the polypeptides which make up the fibres and the penton base and hexon capsomeres, there are four other hexon–associated proteins and four core proteins. However, as many as 15 distinct polypeptides have been demonstrated by SDS–polyacrylamide gel electrophoresis in various types of HAd. (Maizel *et al.*, 1968; Weber *et al.*, 1977) The adenoviruses are double–stranded DNA viruses and possess a genome of approximately 23 x 10^6 daltons, varying slightly according to the serotype.

Pathogenically, HAd3 is most commonly associated with pharyngoconjunctival fever in young children (Bell *et al.*, 1955) as well as certain acute respiratory diseases.

Figure 1. Icosahedral structure of adenoviruses. Number group 1-6 identify the hexon capsomere. Number group 1-5, plus fibre, identifies the penton.


1
2
1
2
3
5
4
3
6
5
4

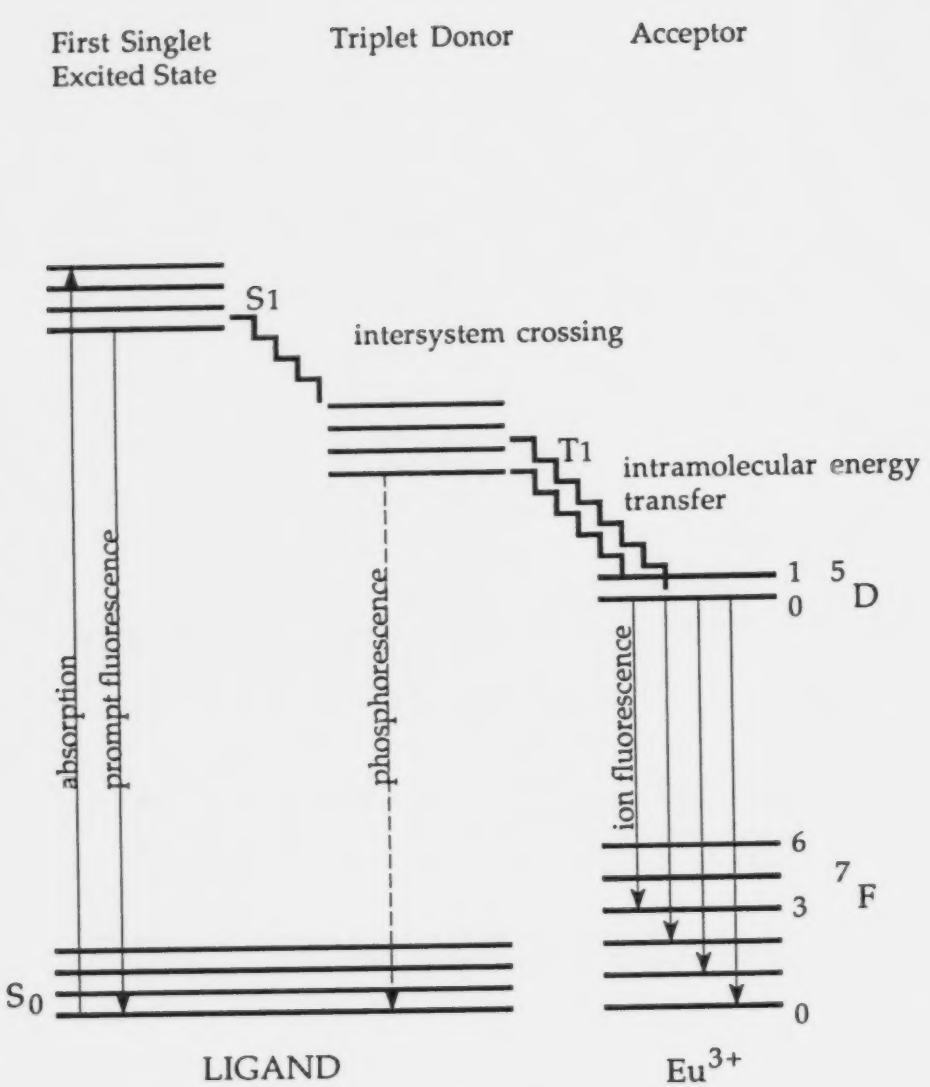
HAd3 is best cultured in human cell lines particularly HEp-2, HeLa, and KB; however, Vero cell sub–lines or clones may be used. The cytopathic effect (CPE) of adenoviruses generally consists of the rounding and clustering of infected cells into grape–like structures. Normal cultures of HAd3 in HEp-2 cells reach CPE in 3-5 days; this may be shortened to 24 hours if the inoculum is increased or if the virus is centrifuged onto the cell monolayer.

HAd3 was chosen as a model virus to test the assay as it is inocuous, fairly well understood, and is reasonably easy to culture.

C. Fluorescence

Luminescent molecules have the ability to absorb energy and then emit photons. Please refer to Figure 2. The absorbed energy (from chemical reaction or radiation) induces the molecules' outer–orbital electrons to change from the ground state (S_0) to a higher electronic state (S_1, S_2, S_3, etc.) From this excited state, the molecule may return to ground by nonradiative processes (heat), or by radiative means (fluorescence), directly back to the ground state; the molecule may undergo inter–system crossing from the single to the triplet system where it may reach the lowest triplet state (T_1). From this state it may return to the ground state by means of a spin–forbidden transition (T_1 to S_0), resulting in phosphorescence. The loss of energy in luminescence, seen as the differences between the excited and emission wavelengths, is known as the Stoke's shift. The energy dissipated over the lifetime of the excited state before it returns to ground state indicates the extent of the Stoke's shift. The percentage of molecule

Figure 2 . Schematic diagram of the electronic energy levels and energy transitions in a fluorescent organic molecule and the radiative processes of the chelate leading to Eu metal ion fluorescence (after Soini & Lövgren, 1987).


First Singlet
Excited State
Triplet Donor
Acceptor
S1
intersystem crossing
T1
intramolecular energy
transfer
1
0
5
D
absorption
prompt fluorescence
phosphorescence
ion fluorescence
6
3
0
7
F
S0
LIGAND
Eu3+

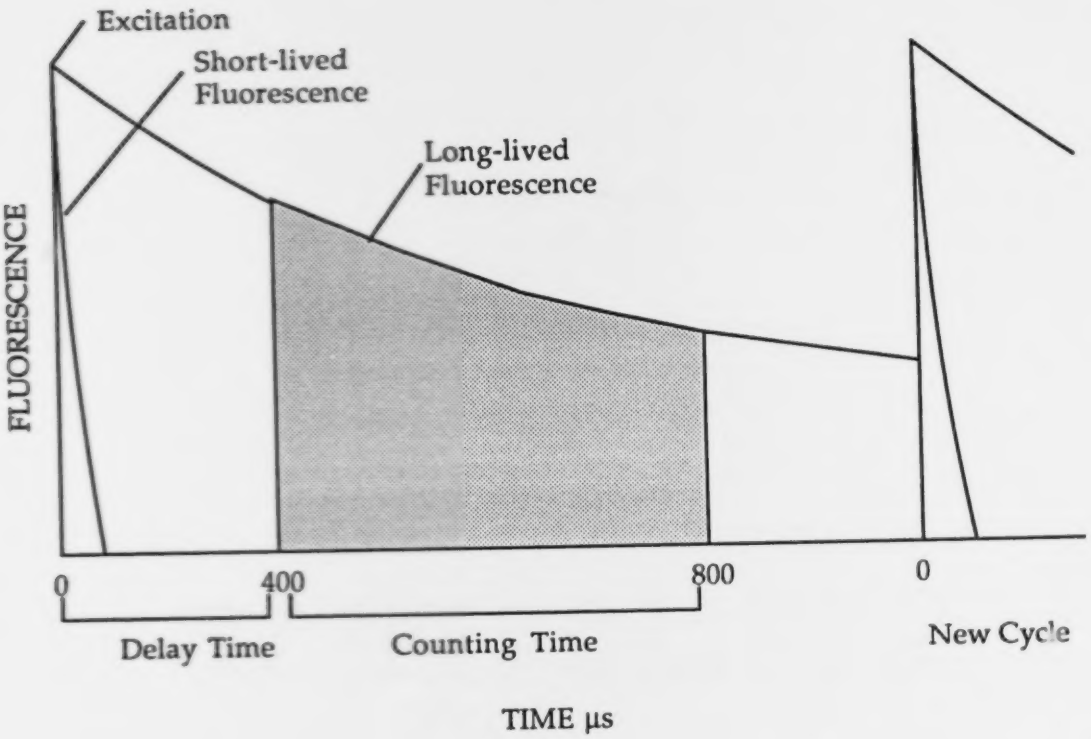
which actually emit light is known as the fluorescence quantum yield (Horrocks, 1993, Soini & Lövgren, 1987).

The group of elements know as the lanthanides (particularly europium, samarium, and terbium) are characterized by having large Stoke's shifts. In fluorescent molecules, a typical Stoke's shift is about 30 to 50 nm; in the lanthanides, that shift may be as large as 200 nm (^5_0D to ^7_2F). The lanthanides are also characterized as having long fluorescent decay times and, despite having a relatively low fluorescence quantum yield, are excellent fluorescent labels when used with the technology known as time–resolved fluorimetry (TRF) (Hurskainen *et al.*, 1989, Horrocks, 1993).

D. Principles of time–resolved fluorimetry

The examination of emission spectra at different intervals of fluorescent decay is know as time–resolved fluorimetry. Immediately after excitation, Raman and Rayleigh scattering as well as short–decay fluorescent factors, are prevalent (Soini & Lövgren, 1987); these are usually considered background interference. Time–resolved fluorescence technology relies on the phenomenon that excited lanthanide ions have long decay times (approximately 50 to 1000 μs), extending much later than these initial spectra. This long–lived decay allows measurement of the lanthanide fluorescence after (usually 400 μs) the short–lived fluorescence of background signals has faded. The background signals may originate from dust particles, compounds incorporated into the assay (for example, bovine serum albumin), or the plasticware used in the assay itself. This system allows the

Figure 3. Measurement principle of time–resolved fluorescence for europium. The sample is excited at 314 nm with a short light–pulse (about 1 ns). Background interfering fluorescence decays within 400 μs, after which Europium fluorescence decay is measured at 613 nm during the 400 μs to 800 μs time interval. The cycle repeats after 1 second, at the next well.


Excitation
Short-lived
Fluorescence
Long-lived
Fluorescence
FLUORESCENCE
0
400
800
0
Delay Time
Counting Time
New Cycle
TIME µs

interfering background noise, typical of many fluorescent assays, to be ignored (Figure 3). This ability to discriminate between background and probe fluorescence permits greater sensitivity than obtainable with the conventional measurement methods of the short–decay organic fluorescent probes.

E. Chelates

Ions of lanthanides are unique in that they possess an unfilled 4F electron shell which has the capacity to hold up to 14 electrons. Salts of lanthanides alone are only weakly fluorescent; however, fluorescence may be enhanced by binding the lanthanide ion to appropriate organic ligands. Weissman (1942) found that β–diketone complexes (R_1-CO-CH_2-CO-R_2) of trivalent europium, terbium, and samarium were highly fluorescent when excited by near–UV light. Intra–4F electronic transitions within the lanthanide ions occurred whenever their bound ligands were excited. It was discovered that it was the ligand of the ligand–lanthanide complex that was absorbing the excitation energy, and from this he postulated that an internal energy transfer from the ligand to the lanthanide ion was occurring, permitting fluorescence of the lanthanide ion. In 1962, Crosby *et al.* fully explained this phenomena.

Upon excitation of the chelate to a vibrational level of the first excited singlet state (S_0 to S_1) the molecule may decay radiatively from S_1 to S_0 (prompt ligand fluorescence) or transfer to one of the triplet states, T (Figure 2). From the triplet state, the molecule may

phosphoresce via a spin–forbidden transition (T to S_0), or the excitation may be transferred to an appropriate 4F energy level of the central lanthanide ion. Only certain excited energy levels of the lanthanide ion may result in fluorescence, these are therefore designated the "resonance levels" (Soini & Lövgren, 1987).

Non–radiative decay (S_1-S_0) and phosphorescence (T-S_0) are competing pathways to fluorescence. When these two pathways are minimized, ideal decay of the fluorescent lanthanide occurs. Chelating a lanthanide ion is essential in maximizing the fluorescence. Chelates of the lanthanide ions Sm^{3+}, Eu^{3+}, Tb^{3+}, and Dy^{3+} exhibit excellent decay characteristics with strong fluorescence and weak interference of the competing pathways. This group are efficient at intramolecular energy transfer, because their resonance levels are close to the triplet levels; as well, they have a low incidence of non–radiative decay events, due to ideal spacing of the outer energy levels. (Whan & Crosby, 1962; Crosby, 1966)

F. Factors which influence fluorescence

Generally, the longer the decay time, the more intense the fluorescence. The ability of the ligand to receive energy and to transfer it to the resonance level of the lanthanide ion, as well as the extent of the competing, non–radiative decay pathways will control the intensity of the fluorescence. The nature of the particular lanthanide ion, the characteristics of the ligand, the ion–ligand bond, loosely associated molecules, and the solvent environment will all affect the intensity of fluorescence. In addition to these factors, other qualitative conclusions

regarding the energy transfer between ligand and lanthanide, and fluorescence intensity have been made in detail elsewhere (Filipescu, *et al.* 1964; Sinha, 1971). Overall, ligand–ion energy transfer governs the absorption and emission properties of the ion.

In 1964, Halverson *et al.* proposed the concept of the "insulating sheath". In principle, it protects the lanthanide ion from the quenching effects of its environment. De–excitation of molecules in excited energy states may occur via many undesirable nonradiative mechanisms. The rate of transfer depends on the coupling between the energy donor and acceptor, as well as the number of acceptor states. Generally, the stronger the bond between donor and acceptor, the better the energy transfer. Halverson *et al.* (1964) write, "Thus, in order to minimize the radiationless transfer, it would appear desirable to enclose the "electronic energy–rich moiety" (potential donor) in a sheath which insulates the donor (maintains minimum distance) from better energy acceptors in its environment."

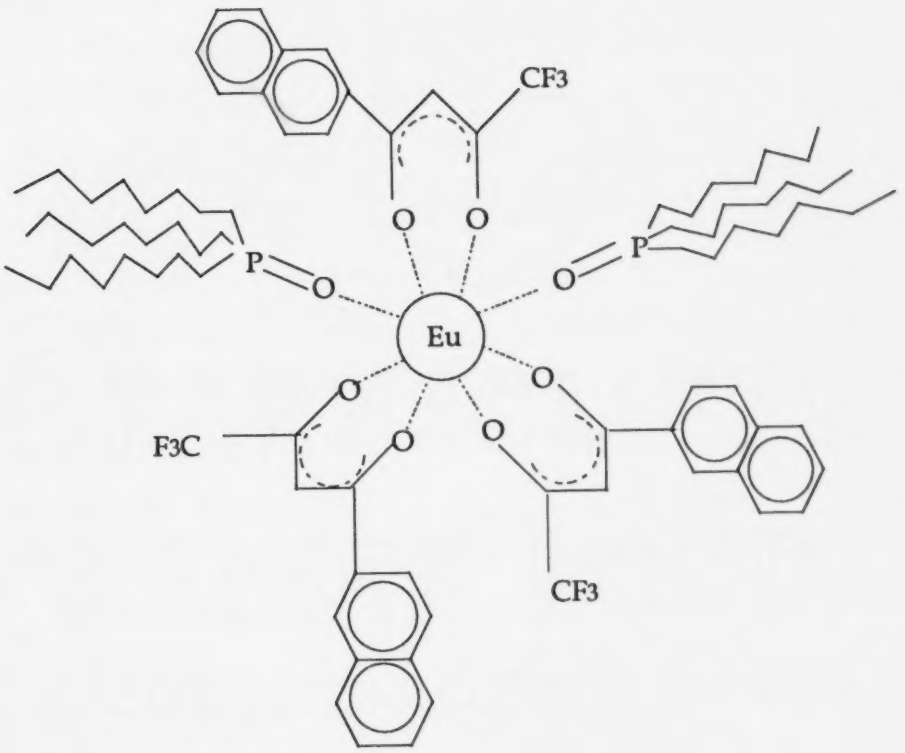
In the case of β–diketone chelates of lanthanide metal ions, the chromophore is attached to the ion via oxygen atoms. The sheath must be able to prevent the proximal approach of accepting systems, quenching impurities, and undesirable solvent components and so must have bulky, interfering groups attached to its carbon skeleton. The sheath itself cannot have these accepting and available energy levels, so, typical bulky groupings include saturated hydrocarbons, or fluorinated or chlorinated hydrocarbons.

The bulky insulating sheath may be attached to the lanthanide–chelate by using synergistic agents, such as a so–called Lewis base. The lanthanides are tripositive metal ions and are held in place via six

oxygen atoms of the β–diketone. However, lanthanide ions prefer seven to nine oxygen atoms in their outer orbital. The synergistic agents are neutral molecules such as trioctylphosphine oxide (TOPO) with a lone oxygen atom available to the lanthanide ion. TOPO also has a bulky saturated hydrocarbon tail which is directed away from the lanthanide metal ion, contributing to the insulating sheath (Figure 4).

In order to apply lanthanides as labels, they must fulfill two requirements (Soini & Lövgren, 1987). First they must be stably bound to the ligand, and, secondly, they must form a highly fluorescent complex with the ligand. Normally, these two requirements are tied into one compound; however, DELFIA® separates these two functions following the "insulating sheath" principle. The lanthanide ion is stably bound to the labelling compound under the buffered conditions present at the time of labelling. This stability is dependent upon the pH of the surrounding solution. When "Enhancement Solution" is added, the pH is lowered (2.8 - 3.5) and the stability of the chelating complex is decreased, and with a so–called splitting detergent, such as Triton X100, causes the dissociation the lanthanide ion into solution (Hemmilä & Dakuba, 1982). The "Enhancement Solution" also contains a synergistic agent and a β–diketone suitable to the particular lanthanide ion used. The result is a highly fluorescent micellular structure which may be read by TRF.

Figure 4. An hypothesized form of the europium chelate, consisting of an europium ion, three 2-napthoyltrifluoroacetone, and two tri(*n*-octy) phosphine oxide molecules (after Lövgren *et al.*, 1985)


CF3
O
O
P
O
O
P
Eu
O
O
F3C
O
O
CF3

G. Development of time–resolved fluorimetry technology

The concept of using lanthanides and their ligands to study DNA was first introduced by Stern & Steinberg (1953). Its potential with other substances of biological interest was proposed by Stern (1956). Leif *et al.* (1976) discussed the possibility of labelling antibodies with lanthanide chelates to detect various cellular antigens. Leif was one of the first to fully propose the chemistry of a fluoroimmunoassay. Soini & Hemmilä (1979) presented a review of the potential of this technology. Since then, improvements to chelating agents, assay designs, and detection systems have made time resolved fluoroimmunoassays (TRFIA) a common technique in the clinical laboratory.

While immunoassays are currently a technical mainstay in clinical laboratories, DNA hybridization (DNA probing) methodologies are emerging as promising new options in the field of laboratory medicine (Wolcott, 1992). The specificity offered by DNA hybridization assays is unrivaled by immunoassay. However, immunoassays may not lose their importance in diagnostic work; they are essential in detecting the products of infection when few pathogenic organisms are present or if a group of similar organisms are suspect, rather than a particular unique strain. The progression toward the use of DNA probing is facilitated by the fact that there are many similarities in assay designs and labelling systems between the two techniques. New DNA hybridization assays can only serve to complement the immunoassays already in service (Diamandis, 1990).

In immunoassays, non–isotopic labelling systems, such as time–resolved fluoroimmunoassays, are successfully replacing the traditional radioactive labels (Halonen, *et al.* 1983, Hemmilä, 1986, Pettersson, 1990, Siitari, 1990) This is also becoming the trend in DNA hybridization assays. The challenge is to provide a highly specific, highly sensitive, and automated assay. The biggest advantage of non–radioactive assays is their safety; however, radionuclides remain dominant as many alternative labelling systems are still less sensitive.

H. Development of time–resolved fluorimetry in DNA hybridization assays

Since the 1950's, when the original concept of using time–resolved fluorimetry to detect DNA was introduced, the bulk of the research performed has switched to the field of immunoassays. The return to DNA hybridization assays using TRF occurred in the late 1980's. The first major advance was a natural step, combining DNA hybridization with immunological reactions (Syvänen *et al.*, 1986). The hybridization probe used to detect the DNA was labelled with a hapten to which antibodies were made. This probe was subsequently recognized by a two–step immunoassay; the second antibody was labelled with europium.

Also that year, Viscidi and his group published a method of simply labelling nucleic acid probes with biotin (Viscidi *et al.*, 1986) This technique advanced TRF DNA hybridization assay in that the biotin–labelled probes could be reacted with europium–labelled streptavidin which could be read by TRF (Dahlén, 1987; Dahlén, *et al.*,

1987). Dahlén made further contributions in advancing TRF technology by proving that DNA probes could be directly labelled with europium using transamination (Dahlén *et al.*, 1988)

Hurskainen, working with Dahlén's group, also advanced Dahlén's work by implementing transaminated, europium–labelled probes into a DNA hybridization assay (Hurskainen *et al.*, 1991) This technique involved sandwiching the desired DNA between two probes, one directly bound to the surface of the 96–well microtitre plate, the other labelled with europium and the plates were read by TRF.

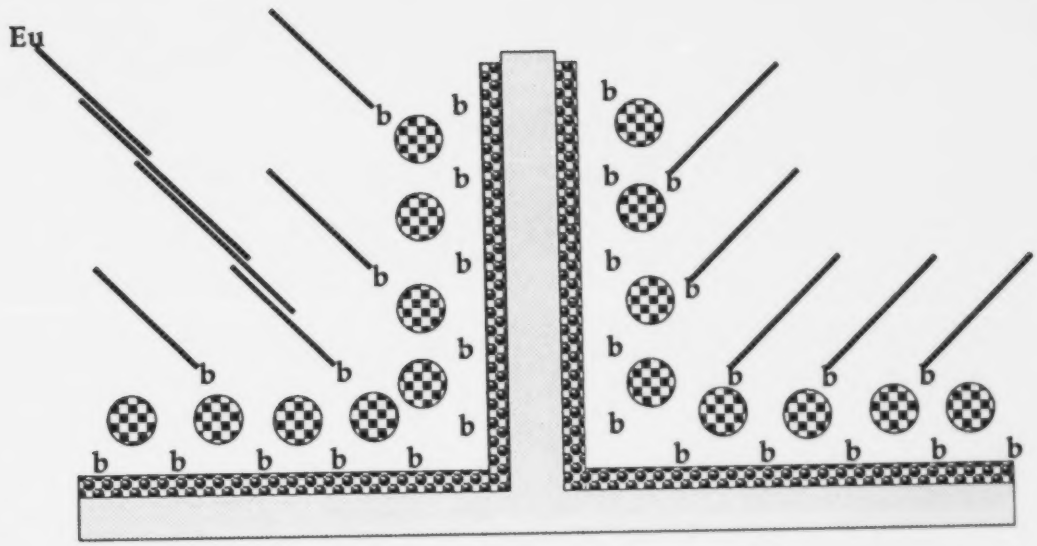
That same year, Dahlén and his group introduced the use of PCR in conjunction with sandwich hybridization and TRF (Dahlén *et al.*, 1991). His technique involved a complicated but useful means of attaching the DNA hybridization sandwich to the 96–well microtitre plate. The wells were first coated with biotin–labelled BSA and then the biotin was saturated with streptavidin. The sample was subjected to PCR using two unlabelled primers. Following this amplification of the desired section of DNA, the product was boiled and then two probes, situated inside the amplified section, were added and hybridized. One probe was labelled with biotin via a diaminohexane–modified deoxycytidine at the 3′ end; the second probe was labelled with europium via a 35-40 diaminohexane deoxycytidine tail. These two probes sandwiched the amplified DNA fragment and attached it to the capture mechanism on the plate via the interaction between the biotin and the streptavidin; streptavidin may theoretically bind up to four biotin molecules (Chaiet & Wolf, 1964).

The techniques developed by these two groups have contributed greatly to this research. The labelling protocols of Hurskainen's group

(1991) and the capture mechanism of Dahlén's team (1991b) were instrumental in the assay design. Although Dahlén chose to amplify the desired template DNA, my assay has been designed without PCR. It is well understood that PCR is highly susceptible to cross–contamination by minuscule amounts of carry–over DNA; and, although the characteristics of europium provides immunity to background interference, the assay is still highly susceptible to contamination by delinquent europium aerosol particles. Because of the sensitivity of europium itself, the potentiality of contamination by combining PCR represents an unacceptable risk of obtaining false positive results.

The 96–well plates were prepared as Hurskainen *et al.* (1991) described (Figure 5). This format provides an adaptable matrix upon which any sample may be inspected for desired DNA sequences. Microtitre plates coated in this way allow any organism's DNA to be captured, provided one of the sandwiching probes is labelled with biotin. For each application, the probes may be simply selected and easily labelled. An advantage is that many plates may be prepared at once and stored for later use; this design lends itself well to use in a kit format. The actual hybridization takes place outside the microtitre plates, in a temperature–controlled environment; the capture mechanism simply provides a support for reading in the fluorimeter. This also permits the definition of the hybridization characteristics required for each particular sample probed. Many different samples may be read simultaneously on one plate, saving time and expense. Although the assay itself is built around a viral model, it was developed to be flexible in its application.

Figure 5. Diagrammatic representation of the capture mechanism coated onto each well in the 96–well microtitre plate. Bovine serum albumen (BSA) is labeled with biotin and then coated onto the surfaces of the well. The biotin is then saturated with streptavidin. **A.** Positive reaction: europium–labelled (**Eu**——) and biotin–labelled probe (**b**——) are bound to the HAd3 DNA target (═) and captured by the biotin streptavidin interaction. Europium signal is read. **B.** Negative reaction: no target DNA and therefore only biotin–labelled probe is captured with no europium signal generated.



A

B

b biotin-labelled BSA

streptavidin

MATERIALS AND METHODS

A. Virus and tissue culture cell lines

Human adenovirus type three (HAd3) was obtained from E. Norrby, Stockholm, Sweden. HEp-2 cells (ATCC CCL23, derived from human larynx epidermoid carcinoma) were used to culture the virus and were obtained from ATCC (Rockville, Maryland).

B. Cell culture media and reagents

The growth medium for monolayer cultures of HEp-2 cells was Autopow® Minimal Essential Medium (MEM) with Earl's salts (Flow Laboratories, ICN Biomedicals Inc., Mississauga, Ontario) supplemented with 1 % L–glutamine, 0.2 % sodium bicarbonate, 2.5 % bovine serum (CS) (Gibco® Laboratories, Life Technologies Inc. Grand Island, New York), and 100 IU of penicillin and 0.1 mg of streptomycin sulfate per ml.

C. Cell culture

HEp-2 cells were grown in sealed, 150 cm^2 glass culture vessels (Blake bottles) or polystyrene flasks (Corning Glass Works, Fisher Scientific). The cells were maintained at 37 °C and the medium was changed when required (about every 3 to 5 days) as indicated by the colour of the medium and the appearance of the cells by microscopic

inspection. When the culture medium needed to be replaced, the medium was decanted, the cells were washed with 50 ml PBS (Section Z), and 100 ml of fresh medium was added. When 95 % confluency was reached, cultures were passaged by decanting the medium, gently washing the cells twice with 50 ml PBS and then detaching the cells with 2 ml 0.25 % w/v trypsin. The trypsin was allowed to act on the cells for approximately 2 minutes at either room temperature or at 37 °C, depending on the tenacity of the cells. Cells were diluted to approximately $1x10^6$ cells/ml in 100 ml of medium and transferred to fresh 150 cm^2 culture vessels and sealed. The cells were grown to 80 % confluency at 37 °C if they were to be infected with HAd3 or further incubated to passaging concentration. Sufficient clonal cultures were assured throughout the experiment by periodically freezing actively multiplying cells.

D. Freezing HEp-2 cells

Healthy cultures with low passage numbers were chosen for freezing. When the cells had reached 70 % confluency, they were washed two times with 50 ml PBS and detached from the culture vessels by incubating with 2 ml trypsin for two minutes at 37 °C . The loose cells were collected with 5 ml of medium/tissue culture bottle. The cell suspensions were pooled and centrifuged at 800 Xg for 20 min at 4 °C in a GPR centrifuge using a GH-3.7 rotor (Beckman Instruments, Mississauga, Ontario). When the centrifugation was complete, the supernatant was discarded and the cell pellet was thoroughly re-suspended in 5.75 ml MEM, 5 % fetal bovine serum. Dimethyl

sulfoxide (DMSO) was added to a final concentration of 11 % (v/v) and mixed gently. The cell suspension was dispensed in 1.5 ml aliquots in 1.8 ml cryotubes (Nunc, Gibco BRL, Burlington, Ontario) and capped lightly. The cells were frozen for 1-2 h at -20 °C, then transferred to -70 °C for another 1-2 hr, then to the gas phase of a liquid nitrogen storage container overnight, and finally to the liquid phase of the container, -196 °C. This gradual freezing process prevented the cells from bursting by sudden freezing. One month after freezing, a representative cryotube was rejuvenated to test the viability of the frozen cells. The HEp-2 cells were tested for mycoplasma contamination at monthly intervals.

E. Virus culture

The growth medium for HAd3 virus culture was as described in Section B., but with 5 % fetal bovine serum (FBS) (CELLect® Gold, Flow Laboratories, ICN Biomedicals Inc., Mississauga, Ontario). Virus culture was performed at 37 °C.

Cultures of HAd3 were created by first infecting a 25 cm^2 plastic tissue culture flask with the original virus stock and using the subsequent harvest as seed stock for the remainder of viral propagation operations. A healthy HEp-2 culture at 80 % confluency was gently washed with PBS and infected with HAd3 at a multiplicity of infection (MOI) of 0.1 in 5 ml PBS. The infected cells were incubated for one hour at 37 °C to ensure adsorption of the virus onto the monolayer. The flask was periodically rocked to prevent the cells from drying out

and to evenly distribute the inoculum. After infection, FBS–supplemented culture medium was replaced.

As large quantities of HAd3 virus were required, cells to be infected with virus were cultured in 2 L glass roller bottles (Bellco, Montreal, Quebec). As these culture bottles were cylindrical in shape, cells could be grown on the entire inner surface of the flask. The roller bottles were rotated overnight with 5 ml FBS to thoroughly coat the inner surface and to enhance cell attachment. Cells were maintained as described in Section J, but because of the larger surface area of the roller bottle, appropriate confluency took longer to reach and the medium was changed more frequently.

When the cells had grown to 80 % confluency, they were ready for viral infection. The medium was discarded and the cell monolayer was gently washed with 50 ml of PBS. The live virus was introduced at 0.1 MOI in 10 ml PBS and incubated, rotating, for 1 h at 37 °C. A total of 20 roller bottles were used to produce the virus stock.

F. HAd3 whole virus purification

A single virus stock was used for the entire course of the experiments. Twenty roller bottles of HAd3 infected HEp-2 cells were produced. The infected cells were then washed off the glass with vigorous swirling with 25 ml of PBS. The wash suspensions were centrifuged at 800 Xg in a Beckman GPR Centrifuge using a GH-3.7 rotor for 30 min at 4 °C. The supernatant was discarded and the cell pellets collected and pooled. The cells were disrupted by repeatedly freezing at -70 °C and thawing. The non–ionic detergent, sodium

deoxycholate was added to the cell fragments to a final concentration of 0.2 %. The cell membrane debris was removed from the mixture by gently shaking for 60 min at room temperature and then centrifuging at 800 Xg. The supernatant was collected and layered onto a pre–formed non–linear gradient of cesium chloride.

The non–linear gradient was prepared in a SW-28 Ultra–Clear™ centrifuge tube (Beckman) by pouring the least dense layer first and adding each subsequent denser layer below the previous one using a Pasteur pipette inserted to the bottom of the centrifuge tube; the lighter layer was pushed up by the next denser one with minimal mixing of the layers. The completed gradient consisted of 5 ml of each of the following densities, bottom to top: 1.4000 g/ml (6.4000 g CsCl/ml PBS); 1.3200 g/ml (4.9300 g CsCl/ml PBS); and 1.2000 g/ml (3.0000 g CsCl/ml PBS).

The completed gradient was centrifuged in a Beckman XL-90 ultracentrifuge using a SW-28 swinging bucket rotor at 70,000 Xg for 90 min at 4 °C. The virus was identified as a bluish white band and was drawn off using a syringe and 21 G needle. This preparation was mixed with cesium chloride of a buoyant density of 1.3390 g/ml (5.1600 g CsCl/ml PBS) in a 14 x 89 mm Ultra–Clear™ centrifuge tube (Beckman) and was centrifuged to equilibrium at 100,000 Xg for 20 h at 4 °C in a Beckman SW-41 swinging bucket. The virus, identified as a fine bluish–white band, was again drawn off using a syringe and a 21 G needle.

G. Drop dialysis

When HAd3 virus was required for an experiment, an aliquot was dialysed by drop dialysis (Marusyk & Sergeant, 1980). The fluid against which the virus was to be dialysed, was poured into a sterile, 35 mm plastic petri dish. Either sterile, deionized water or Sodium acetate, pH 5.2 was used. A 0.05 µm pore size Millipore VM (Millipore Corporation, Mississauga, Ontario) filter was carefully placed, shiny-side up, onto the surface of the fluid. Up to 200 µl of virus stock could be added to the dry upper surface of the floating membrane filter. The plate lid was replaced to prevent evaporation and the dialysis was permitted to proceed for 20-30 min. When dialysis was completed, the virus drop was carefully pipetted off the surface of the membrane filter.

H. Viral DNA extraction

Viral DNA was extracted from purified HAd3 virions. An aliquot of dialysed virus particles was placed in a 1.5 ml microfuge tube and subjected to five repeated cycles of freezing at -70 °C and boiling for 10 min. The viral proteins were extracted by adding an equal volume of phenol/chloroform/isoamyl alcohol (25: 24: 1) to the disrupted virus suspension and inverting repeatedly. The mixture was centrifuged in a Microfuge E™ (Beckman) microfuge for 5 min. The aqueous layer was carefully removed and transferred to a new microfuge tube. The addition of phenol/chloroform/isoamyl alcohol was repeated. The mixture was again centrifuged and the aqueous layer was removed.

This procedure was repeated a third time or until the aqueous layer interface was clear. Residual phenol was removed by adding an equal amount of chloroform/isoamyl alcohol solution (24: 1) and inverting repeatedly. The mixture was centrifuged for 5 min and the top aqueous layer removed and retained in a new microfuge tube. This procedure was repeated if carry–over was suspected.

I. Ethanol precipitation

To extract the DNA from the aqueous solution, one–tenth volume of 3 M sodium acetate, pH 7.0, and 2.5 volumes of ice–cold 95 % ethanol or an equal volume of isopropyl alcohol were added. If the DNA was separated from nucleotides, 10 M ammonium acetate was added to a final concentration of 2.5 M instead of using sodium acetate. The mixture was mixed well by inversion and incubated for 30-60 min at -20 °C . The tube was then centrifuged for 15 min to sediment the precipitated DNA. To wash the DNA pellet, 1 ml of ice–cold 70 % ethanol was added and the mixture was incubated for 30 min at -20 °C . The DNA pellet was then centrifuged for 15 min and the supernatant was decanted. Any residual ethanol was evaporated by incubation in a heating block at 37 °C overnight.

J. Synthesis of primers and probes

A PCR–Mate™ DNA Synthesizer, Model 391 (Applied Biosystems, Mississauga, Ontario) was used to synthesize the PCR primers and viral probes used in the development of the assay. Small

scale (0.2 μM) columns were used and synthesis was carried out using cycle three (reagent–conserving cycle) and "trityl on" configuration (the 5′ terminus is protected with a dimethoxytrityl group) if purification was via oligo purification cartridge (OPC™), or "trityl off" (5′ terminus not protected) if the purification of the product was by polyacrylamide gel electrophoresis. The purification method used was determined by the quantity of DNA required; OPC™ purification generally yields only 1 - 5 OD_{260} units of DNA. Polyacrylamide gel electrophoresis was used when large quantities of pure oligonucleotide was needed.

All columns and phosphoramidites were purchased from Applied Biosystems. The synthesis reagents were purchased from Applied Biosystems and Glen Research (Sterling, Virginia). The synthesizer was operated according to the manufacturers instructions.

When the synthesis was complete, the column containing the DNA product was removed. A 5 ml syringe was attached at each end of the column and 3 ml of fresh, concentrated ammonium hydroxide was flushed, back and forth, three times, through the column to loosen the matrix. The ammonium hydroxide was then pushed through the column in 0.5 ml increments every 30 - 60 min to simultaneously decyanoethylate and cleave the DNA from the support matrix. With the DNA dissolved in the ammonium hydroxide, the liquid was transferred to an Opticlear® 8 ml glass vial with a teflon–lined screw cap (Kimble, Fisher Scientific). The suspension was incubated overnight at 55 °C to remove the base protecting groups.

Following overnight deprotection, the liquid was transferred in 1 ml aliquots to 1.5 ml microfuge tubes. Caps were removed from other microfuge tubes and pierced 5 or 6 times with a sterile 18 G

needle to cover these tubes. This permitted the evaporation of the ammonium hydroxide and afforded some protection from aerosol contamination. The tubes were placed in a Speed Vac Concentrator (Savant Instruments Inc., Fisher Scientific) and centrifuged under vacuum and gentle heat until dry.

The dried DNA was redissolved in sterile deionized H_2O and then precipitated by ethanol precipitation followed by 70 % ethanol wash to remove any residual salts or contaminants of synthesis as described in Section I. The DNA pellet was again dissolved in sterile deionized H_2O and an aliquot was taken to read the optical density at 260 nm to determine the yield of the synthesis.

K. Oligonucleotide purification by polyacrylamide gel electrophoresis and UV shadowing

Due to the great quantities of pure DNA required to test the assay, oligonucleotides synthesized as probes were purified using polyacrylamide gel electrophoresis and UV shadow analysis. Because of occasional failure during synthesis, n-1, n-2, etc., products contaminate the final oligonucleotide product. These smaller fragments are less specific probes and may also interfere with subsequent DNA manipulations. The procedure used was adapted from Applied Biosystems, Inc., User Bulletin #13–Revised (1987).

The DNA probes were synthesized on the PCR–Mate™ DNA Synthesizer, Model 391, in the "trityl on" configuration, removed from the columns, and deprotected, as described in Section J. The crude, dried product was dissolved in sterile, deionized water to a

concentration of approximately 40 µg/µl and mixed 1: 1 with sterile glycerol.

A 15 % polyacrylamide gel was prepared with 4 M urea and 19:1 acrylamide/bis ratio. The gel was cast into a Protean II vertical electrophoresis sandwich apparatus (BioRad, Mississauga, Ontario) using 1.5 mm spacers. The glass plates, the spacers, and the comb were all cleaned well and rinsed with 95 % ethanol to prevent bubbles forming on the glass and to allow uninterrupted polymerization of the acrylamide. The smaller glass plate was coated with dichloromethylsilane (Fluka, Caledon, Edmonton, Alberta) to allow easy removal of the gel after completion of the run. The comb selected had five, 20 mm teeth and was inserted about 2 cm into the gel. When the gel had polymerized, the assembly was attached to the Protean II cooling core and placed inside the Protean cell. The cell and the upper chamber were filled with 1 X TBE (Section Z). The comb was removed and the wells were flushed with buffer using a pasteur pipette to remove any unpolymerized acrylamide solution. The first lane was loaded with running buffer (Section Z) only; into each of the remaining wells, up to 200 µl of crude DNA/glycerol mix was loaded (no running buffer was added with the DNA, as the dye could interfere with UV visualization of the gel.)

The samples were separated on the gel at 30 mA for approximately 3 hours or until the dye front had reached the bottom of the gel. The gel apparatus was then dismantled and the gel was carefully removed from the glass sandwich. The gel was placed on a sheet of Saran Wrap plastic film (DowBrands Canada Inc.). To visualize the DNA in the gel, UV shadowing was utilized. A fluorescent screen was

prepared by taping four, 5 x 20 cm Baker–Flex flexible thin–layer chromatography (TLC) cellulose CM sheets side–by–side onto a 1 ft^2 piece of cardboard. The gel on the Saran Wrap was placed onto this device and illuminated from above with a short wave ultra–violet Model UVG-54 Mineralight lamp (Ultra–violet Products Inc., San Gabriel, California) emitting 254 nm light. The TLC sheets provided an even fluorescent background against which the DNA in the gel cast a shadow, indicating the positions of the complete DNA product and the incomplete oligonucleotide fragments.

The shadow band corresponding to the desired product was excised using a scalpel blade and placed into a 1.5 ml microfuge tube. The gel slab was crushed using a 200 µl pipette tip. To elute the DNA, 500-1000 µl of sterile deionized water was added to the crushed gel slab and incubated overnight in a 37°C water bath. Following incubation, the bottom of the tube was pierced with a red–hot 30 G 0.5 inch needle and set into a 15 ml polypropylene centrifuge tube (Costar Corporation, Sincan, Calgary, Alberta). The tube was centrifuged at 800 Xg in a GPR centrifuge using a GH-37 rotor. The liquid in the 15 ml tube was then collected and passed through a sterile Millex®–GV 0.22 µm Filter Unit (Millipore). This filter material displays low DNA binding and removed any tiny fragments of polyacrylamide gel. The purified oligonucleotide dissolved in this liquid was extracted by ethanol precipitation as described in Section I.

L. Oligonucleotide purification by OPC™

Oligonucleotides which were amino–linked and purified by the oligo purification cartridge method were synthesized on a PCR–Mate™ DNA Synthesizer Model 391 using cycle 1 (optimum yield), in a "trityl–on" configuration (5′ terminus protected), and cleaved and deprotected as described in Section J.

The product was then purified on an oligo purification cartridge, OPC™. The concentrated ammonium hydroxide present in the loading solution protects the oligonucleotide from denaturation but also interferes with the binding of the oligonucleotide to the purification cartridge. The concentrated ammonium hydroxide was diluted with one–third volume of sterile deionized H_2O. The column was then connected to a 5 ml polypropylene syringe. The OPC™ was clamped syringe–up to a laboratory support. The cartridge was prepared by gently flushing 5 ml of HPLC grade acetonitrile, followed by 5 ml 2.0 M triethylamine acetate. The diluted ammonium hydroxide was then gently pushed through the cartridge at a rate of about 1-2 drops/sec. Each of the subsequent steps were performed at this rate. The eluted fraction was saved and reapplied onto the cartridge two more times. The cartridge was then washed with 3 X 5 ml of 1.5 M sodium acetate followed by 2 X 5 ml deionized H_2O. To detritylate the oligonucleotide bound to the cartridge, 5 ml of 2 % trifluoroacetic acid was loaded into the syringe, 1 ml was pushed through and, after a 5 minute interval, the remainder was pushed through. The detritylated, bound oligonucleotide was then washed with 2 X 5 ml of deionized H_2O. The purified oligonucleotide was

eluted with 1 ml of 20 % HPLC acetonitrile in deionized H_2O. The amount of purified product was determined from the OD_{260} units.

M. Primers and probes

Sequences for selection of primers and probes were determined from the IX polypeptide (Sussenbach, 1984). Europium and biotin probes flanked a 320 base region. The europium–labelled probe was a thirty–mer:

5' CAT GGG ATC CAC TGT GGA TGG GAG ACC CGT 3'

and was positioned from 157 to 186 in the IX polypeptide. This probe was called E157. This probe was also synthesized with an amino group attached to the 5′ end, L157. The biotin–labelled probe, B477, was found in position 477 to 448 and was also a thirty–mer:

5' AGA CTT TGC TGT GGC AAC AGC AGA CTC AGT 3'.

This probe was also synthesized with an amino group attached to the 5′ end, L477. The primer used to create the europium–labelled probe by PCR, A210, was at position 210 to 227 and was an eighteen–mer:

5' AGC GTT GAG GAA TTG GCG 3'.

The primer used to create the biotin–labelled probe by unidirectional PCR, A455, was found in position 455 to 475:

5' AGT TGC GTG AGC AAA CTG AGT 3'.

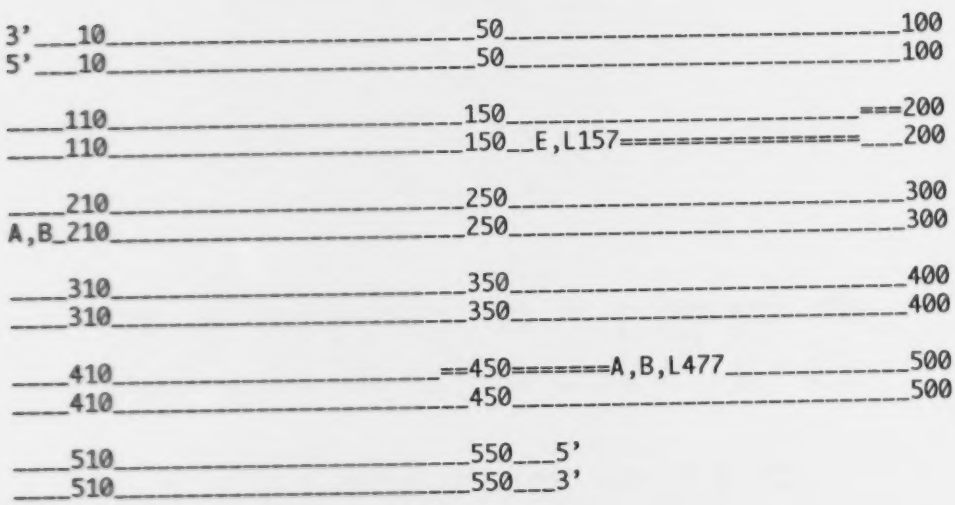
These oligonucleotides are summarized in Table 1. Their distribution on the HAd3 polypeptide IX gene is shown in Figure 6.

Table 1. Oligonucleotides and their sequences.

Oligonucleotide		Sequence
E157		5′CATGGGATCCACTGTGGATGGGAGACCCGT3′
L157	*	5′aCATGGGATCCACTGTGGATGGGAGACCCGT3′
A477		5′AGACTTTGCTGTGGCAACAGCAGACTCAGT3′
B477	‡	5′bAGACTTTGCTGTGGCAACAGCAGACTCAGT3′
L477		5′aAGACTTTGCTGTGGCAACAGCAGACTCAGT3′
A210		5′AGCGTTGAGGAATTGGCG3′
B210		5′bAGCGTTGAGGAATTGGCG3′

* a: Aminolink 2™; ‡ b: biotin–amidite

Figure 6. Position on HAd3 polypeptide IX of primers and probes E157, L157, A210, B210, A477, B477, and L477.


3'___10_____50_____100
5'___10_____50_____100
____110_____150_____===200
____110_____150__E,L157==========___200
____210_____250_____300
A,B_210_____250_____300
____310_____350_____400
____310_____350_____400
____410_____==450=======A,B,L477_____500
____410_____450_____500
____510_____550___5'
____510_____550___3'

N. Polymerase chain reaction

The polymerase chain reaction (PCR) was employed to check the specificity of the probes for the HAd3 DNA. The region chosen for study was the gene encoding the hexon subunit of the viral capsid, specifically polypeptide IX (Sussenbach, 1984). The following ingredients were mixed in 0.5 ml reaction tubes: 2 µl, 2.5 mM dNTP; 5 µl 10X buffer; 2 µl, 50 mM MgCl; 40 pmol each primer (2 µl); 2.5 pmol of target HAd3 DNA; 0.2 µl AmpliTaq® DNA Polymerase (Perkin Elmer Cetus, Rexdale, Ontario) and sterile H_2O to 50 µl. The following cycle temperatures were initiated: Denature, 94 °C, 1 min; anneal 55 °C, 1 min; extend, 72 °C, 2 min. This pattern was repeated for 30 cycles using the GeneAmp PCR System 9600 (Perkin Elmer Cetus). When amplification was complete, the product was evaluated by electrophoresis on a 1 % agarose horizontal gel.

The amount of HAd3 DNA was varied to determine optimal concentration. A titration was performed using standard reagents and 250 pmol, 25 pmol, 2.5 pmol, 250 fmol, and 25 fmol of HAd3 DNA. Optimal template concentration was evaluated subjectively by agarose electrophoresis.

Clinical isolates of various types of adenovirus were obtained from the Virology section of the Division of Microbiology and Public Health of the University of Alberta Hospitals. These isolates were crudely prepared by repeatedly freezing at -70 °C and boiling for 10 min. and then subjected to the same PCR protocol used to test the probes.

O. Agarose electrophoresis

A 1 % agarose gel was prepared by dissolving 1 g of electrophoresis grade Ultrapure™ Agarose (Gibco BRL) in 100 ml 0.5 X TBE. This was accomplished by boiling the solution in the microwave for 1 min and then reducing the power to keep warm for 10 min. The hot solution was allowed to cool to 50 °C, ethidium bromide was added to a final concentration of 0.05 µg/ml, and the mixture was then poured into an appropriate gel tray; the ends sealed with masking tape. A gel comb was suspended above the liquid, 1 cm from one end of the gel such that the bottom of the teeth were inserted to an approximate depth of two–thirds of the gel itself. Any bubbles attached to the comb or on the surface of the liquid were removed with a cotton swab. The gel was cooled and hardened for 30 minutes. When the gel was completely set, the comb was carefully removed and the end seams peeled off. The gel, in its tray, was placed in a horizontal gel apparatus and immersed in 0.5 X TBE. The PCR product was loaded into the submerged wells; 15 µl of sample was mixed with 2 µl of loading dye: 0.4 % bromophenol blue, 5 % glycerol in H_2O. A 1 kb molecular weight marker (Gibco BRL) was also included in the gel beside the PCR samples as a size standard. The DNA in the samples was separated by applying a current across the gel. The DNA was separated in the gel at 100 V until the loading dye traveled approximately three–quarters the length of the gel towards the anode (approximately 60 min.) After separation, the electrophoresis apparatus was dismantled and the gel was then rinsed and destained with tap water for about 15 min. The DNA was inspected by placing the gel on a short wavelength Fotodyne

3-4400 model ultra-violet light box (Fotodyne, Bio/Can, Mississauga, Ontario). The ethidium bromide, intercalating with the DNA, indicated the presence of DNA by fluorescing a bright orange-red colour. The PCR product was identified by its mobility relative to that of the molecular weight standard; the size and mobility of the marker was used to calculate the size of the PCR product.

P. Biotinylation of oligonucleotides

Oligonucleotides to be biotinylated were synthesized with a biotin amidite group attached, with an amino-link attached which was later reacted to attach a biotin, transaminated and biotinylated directly, or created using asymmetric PCR.

Biotin amidite (Applied Biosystems) was used in the fifth phosphoramidite position of the 391 DNA Synthesizer (Applied Biosystems) and the synthesizer was reconfigured to extend the coupling time of the biotin amidite to the strand. Biotin amidite is attached at the 5′ end of the oligonucleotide in a "trityl on" synthesis. The oligonucleotide was purified by OPC™.

To biotinylate using Aminolink 2™, the reagent was used in the fifth phosphoramidite position and the synthesizer was instructed to apply the amino group to the 5′ base. Synthesis was in the "trityl on" configuration and the oligonucleotide was purified by OPC™.

Transamination was employed to create amino groups on the oligonucleotide; however, unlike using Aminolink 2™, trans-amination provides labelling sites over the entire length of the oligonucleotide (Viscidi, *et al.* 1986, Hurskainen, *et al.* 1991). In a small

glass beaker, 0.457 g of sodium bisulfite was dissolved in 1 ml sterile deionized H_2O. With the solution on ice, 1 ml of ethylenediamine, followed by 1 ml concentrated HCl, was added drop–wise. The solution was brought to pH 6.0 by very carefully adding concentrated HCl. The acid was added while the solution was on ice, occasionally transferred to a hot plate to dissolve any seed crystals, and then returned to the ice before adding more HCl. When the solution was brought to the correct pH, the volume was adjusted to 5.0 ml with sterile, deionized H_2O. To complete the transamination solution, 50 µl of freshly made 1 mg/ml hydroquinone in 95 % ethanol was added. One volume (100 µl) of oligonucleic acid (5-25 µg) was mixed with nine volumes (900 µl) of transamination solution. This mixture was incubated 3 h at 42 °C. The transamination mixture was placed in 3500 molecular weight cutoff cellulose dialysis tubing (Spectra/Por®, Spectrum Medical Industries, Inc., Los Angles, California) and dialysed overnight against 3 X 500 ml changes of dialysis buffer C (See Section Z). The transaminated DNA was recovered by ethanol precipitation as described earlier.

To attach biotin to either the aminolinked or transaminated oligonucleotide, the same procedure was used. Approximately 25 µg of pure oligonucleotide was dried in a 1.5 ml Eppendorf tube in a Speed Vac concentrator, resuspended in 1 ml deionized H_2O and vacuum centrifuge–dried overnight. The following morning, 20 µl of freshly prepared 0.1M sodium carbonate buffer, pH 9.0, was added to the dried oligonucleotide with a 100 X molar excess of biotin–amidocaproate N′–hydroxysuccinimide ester. The mixture was mixed gently and incubated at room temperature for 4 hr.

The biotin–labelled oligonucleotide was purified on a Sephadex™-25 NAP 5 column (Pharmacia LKB Biotechnology, Baie d'Urfe, Quebec). The top cap of the column was removed and the excess liquid decanted. The bottom cap was removed and the column was supported, upright, in a laboratory clamp. The gel was equilibrated with 10 ml of sodium phosphate equilibration buffer. The biotinylated oligonucleotide was diluted with equilibration buffer to a volume of 0.5 ml and then loaded onto the column surface. The sample was eluted with 1 ml of elution buffer. The eluent was collected in 0.5 ml fractions and read at OD_{260} to determine which fraction contained the biotinylated oligonucleotide. The biotin–labelled oligonucleotide was recovered by ethanol precipitation.

Unidirectional PCR using oligonucleotides E157, A477, and B455 was used to create a biotinylated probe. A 15 cycle round of PCR using E157 and A477 was first employed to create the 330 base template. Primers were removed by a 30,000 dalton molecular weight cut–off Ultrafree®–MC Filter Unit (Millipore). The PCR product was loaded into the filter cup in a volume of 300 µl with 12 mM Tris, pH 7.6. The unit was centrifuged at 2000 Xg for 4 min followed by two 300 µl washes with 12 mM Tris, pH 7.6. The PCR product was recovered by washing off the membrane with 25 µl of 12 mM Tris, pH 7.6 and used as template for the unidirectional amplification. The polymerase chain reaction was carried out using 15 µl of PCR product and oligo A455 was used at 40 pmol; normal reaction concentrations were used but with 1 µl, 2.5 mM dNTP with biotin–labelled dUTP included with dTTP at a ratio of 1:10. Amplification proceeded for 45 cycles of 94 °C, 30 sec; 40 °C, 30 sec.; and 72 °C, 30 sec. The PCR product was evaluated by

electrophoresis on 20 % polyacrylamide gel and precipitated by ethanol precipitation using ammonium acetate.

Q. Dot blot as quality control

Dot–blotting DNA onto nylon membranes was employed in order to determine if biotin was successfully bound to the oligonucleotide probes. A piece of Hybond™–N Nylon hybridization transfer membrane (Amersham, Professional Diagnostics Inc., Edmonton, Alberta) was cut in a rectangle of approximate size to fit a Minifold™ apparatus (Schleicher & Schuell, Inc., Spectrex Limited, Montreal, Quebec) Two pieces of Bio–Dot® Slot (BioRad) format filter paper were wetted with 2 X SSC and placed onto the filter support plate of the filtration manifold. The nylon membrane was also wetted and then placed on top of the two filter papers. The sample well plate was clamped into place and a vacuum was applied. The wells were washed two times with 100 µl of streptavidin saturation buffer. Samples were diluted 1000X, stepwise in one in ten dilutions in streptavidin buffer and a 100 µl aliquot of sample was pipetted directly into the sample well without touching the pipette tip to the membrane. The wells were washed twice with 100 µl of 2X SSC. The apparatus was dismantled and the membrane allowed to air dry slightly (approximately 10 min). The membrane was wrapped, face–down in Saran Wrap plastic film and transferred to a Fotodyne Model 3-4400 short wavelength ultra–violet light box for 10 min to cross–link the DNA to the membrane. The edges of the membrane were trimmed to encompass only the wells which were used. This membrane was

sealed in a plastic pouch (Scotchpak, Kapak Corporation). A small corner of the pouch was cut off and 2 ml of 1 % Blocking Reagent (Boehringer Mannheim, Laval, Quebec) in PBS was added. The membrane was incubated for 2 h at room temperature to block any interfering sites. After blocking, the membrane was removed from the pouch and rinsed for 5 min with 2 X SSC in a slowly agitating bath.

The membrane was then transferred to another pouch, sealed, and 2 ml horseradish peroxidase–conjugated streptavidin in streptavidin saturation buffer (1:100) was added. The pouch was incubated for 15 min at 37 °C. The membrane was then removed from the pouch and washed in seven, 25 ml changes of TBS. The blot was equilibrated in 50 mM sodium acetate buffer, pH 5.0, for 5 min. Colour developer was made fresh: 10 mg 3-amino-9-ethylcarbazole in 2.5 ml N'N'–dimethylformamide added to 47.5 ml, 50 mM sodium acetate, pH 5.0. Directly before the blot was developed, 50 µl 30 % H_2O_2 was added to this solution. The blot was developed in the dark for 1 to 60 min, depending on the intensity of the colour desired. The colour development was terminated by rinsing with distilled water.

R. Oligonucleotide europium labelling by transamination and PCR

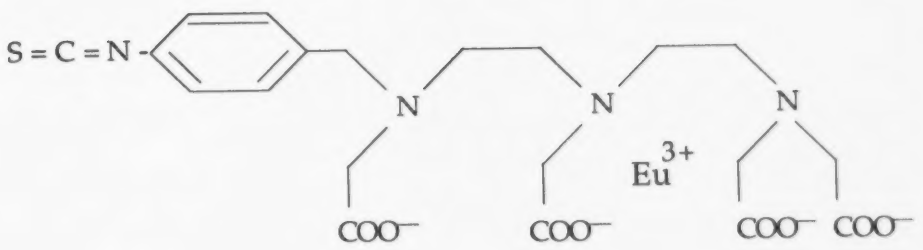
To couple europium to synthesized DNA, transamination, as described for biotin labelling, and PCR were employed.

The labelling agent, a europium–chelate of N^1-(p-isothiocyanatobenzyl) – diethylenetriamine - N^1, N^2, N^3-tetraacetic acid (DTTA) from a DELFIA® Eu–labelling kit 1244-302 (Wallac Oy, Pharmacia) was selected to label the transaminated DNA probes. This

particular kit is marketed as a protein labelling kit; however, the aromatic isothiocyanate group of this compound may be bound to nucleic acid, via transamination of the oligomer (Figure 7). The labelled chelate was coupled to the transaminated oligonucleotide by pipetting 100 µl (5-25 µg) of DNA onto 0.02 mg solid europium chelate. The mixture was allowed to incubate overnight at 4 °C. The following morning, the unincorporated europium chelate was removed by ethanol precipitation using ammonium acetate.

Using a biotin labelled primer adjacent and opposite to the europium–labelled probe, B210, PCR was used to extend and amplify the europium labelled probe (E157–Eu). PCR reagents were mixed as described in Section N. Thirty–five cycles of 1 min at 94 °C, 1 min at 40 °C, and 30 sec at 72 °C was employed. The PCR product was evaluated by electrophoresis using a 2 % agarose gel. The PCR product was denatured by boiling for 5 min and flash freezing; it was then permitted to warm to room temperature. To bind the biotinylated strand 0.2 ng streptavidin was added; the mixture was mixed gently and incubated at room temperature for 5 min. The biotinylated strand was removed by addition of 50 µl of 25:24:1 phenol:chloroform:isoamyl alcohol, mixing and centrifuging for 5 min. The upper aqueous phase was removed and the lower, organic phase was back extracted with 50 µl of 12 mM Tris, 500 mM NaCl. The aqueous phases were pooled and re–extracted with 100 µl of the phenol/chloroform/isoamyl alcohol. The single stranded, europium–labelled probe was precipitated from the final aqueous phase by ethanol precipitation. The product was applied to a 20 % PAG with the original double stranded PCR product to assess the purification. The two were also blotted onto Hybond ™–

Figure 7. The chemical structure of europium–labelling reagent, N^1-(p–isothiocyanatobenzyl)–diethylene–triamine-N^1, N^2, N^3, N^3-tetraacetic acid chelated with Eu^{3+}.


S=C=N-
N
N
N
Eu3+
COO−
COO−
COO−
COO−

N nylon membrane as described in Section P to confirm that the extracted, europium–labelled probe was single stranded.

S. Europium labelling by ligation

Probes were also prepared by ligating, using T4 RNA ligase, the sequence–specific oligonucleotides E157 and A477 to the polycytosine oligonucleotides prepared by transamination and labelled with europium–chelate or biotin, respectively. To ensure that the two oligonucleotides joined together and not to themselves in concatamers, one oligonucleotide was labelled at the 5′ end with a phosphate from ATP using polynucleotide kinase; this oligonucleotide is considered the "donor" in the ligation reaction. Since the oligonucleotides are synthesized with hydroxyl groups at both the 3′ and 5′ ends, the oligonucleotide not altered by polynucleotide kinase would become the "recipient". Upon ligation, the two join as one via the 5′ phosphate of the donor and the 3′ OH of the recipient. All oligonucleotides to be ligated were purified by OPC™ or by polyacrylamide gel electrophoresis to remove incomplete products of synthesis. These shorter fragments acted as contaminants and ligated more efficiently than the desired complete oligonucleotides, resulting in decreased sensitivity of the probes.

The ligation mixture consisted of 1 µl of 10 X reaction buffer, 10 % (v/v) DMSO, 100 U (2 µl) of T4 RNA ligase, 100 pmol of donor and an equal or greater amount of recipient. Since the recipient could not concatamerize to itself, its presence in excess of donor ensured more successful ligation of the two oligonucleotides and competed with the

concatamerization of the donor to itself. The reaction was carried out at 15 °C to constrain the movement of the ends and was permitted to proceed for 24-48 h to ensure the complete joining of the oligonucleotides. The ligation reaction was terminated by boiling for 5 min and the new product was recovered by ethanol precipitation.

T. Tailing of probes with terminal transferase

Probes were also labelled by tailing at the 3′ end with commercially available europium-labelled dCTP (Wallac Oy, Pharmacia) using Terminal Transferase (Boehringer-Mannheim). The reactions were carried out in 0.5 ml microfuge tubes using 0.75 mM $CoCl_2$, 4 µl of 5 X reaction buffer, 2 µl (50 U) terminal transferase, 1.5 µmol Eu-dCTP, 100 pmol ends of oligonucleotide to be tailed, and sterile deionized H_2O to make of the total volume of 20 µl. The Eu-dCTP was always used in large excess of the ends and the mixture was allowed to react for at least 30 - 60 min at 37 °C to ensure that complete tailing was achieved. To terminate the tailing reaction, the mixture was boiled for 5 min and the product was recovered by ethanol precipitation.

U. Biotinylation of bovine serum albumin

Bovine serum albumin (BSA) to be biotinylated was purified on a 12 mm x 47 mm Sephadex G-50 fine gel filtration column. The BSA was eluted with 0.1 M phosphate elution buffer (see Section Z). The eluted product was collected in 1 ml fractions. The optical density of

each fraction was determined at 280 nm using a DU®-65 Spectrophotometer (Beckman). Peak fractions were pooled and dialysed overnight in 3500 molecular weight cutoff cellulose dialysis tubing (Spectra / Por®, Spectrum Medical Industries, Inc. Los Angles, California) against 4 X 500 ml of dialysis buffer A (see Section Z). The BSA concentration in the resultant dialysate was determined by measuring the OD_{280} of the solution. The concentration of the BSA solution was adjusted to a final concentration of 1.0 mg/ml in dialysis buffer A. Biotin was chemically attached to BSA by combining the purified BSA with biotin–amidocaproate N–hydroxysuccinimide ester in DMSO (Hierholzer, *et al.* 1993). The optimal ratio of biotin to BSA was determined. A constant amount of BSA (1 mg/ml) was used and the amount of 1 mg/ml biotin in DMSO was varied. From 0.1 ml to 1 ml amounts of 1 mg/ml biotin in DMSO were combined with the BSA.

The biotinylation reactions were carried out at room temperature with gentle agitation for four hours. The products were dialyzed in 3500 molecular weight cutoff cellulose dialysis tubing against 4 L of PBS (Section Z). The volumes of the dialysates were measured to determine the dilution of the biotinylated BSA.

The extent of biotinylation of the BSA was determined by blotting 0.1 mg of the product onto nitrocellulose membrane (BioRad) using the procedure described in Section Q. The protein–blotted membrane was blocked with 1 % Blocking Reagent (Boehringer Mannheim, Laval Quebec) while agitating at room temperature overnight. The membrane was then sealed into a Scotchpak pouch with 2 ml of 1:100 dilution of HRP–conjugated streptavidin (BioRad) in PBS. The pouch was incubated for 15 min. at 37 °C. The blot was

rinsed in 2X SSC and excess streptavidin was removed by rinsing in 5, 25 ml exchanges of TBS.

Colour development of the blot was performed as described in Section Q.

V. Preparation of microtitre plates

Ninety–six well, flat bottom plates (Polysorp, Nunc) were coated with BSA–conjugated biotin. To each well, 100 ng BSA was added in 200 µl coating buffer. The plates were incubated overnight at room temperature. The following morning each plate was thoroughly washed to remove any unbound protein. Each well was washed ten times using an Immuno Wash 8 (Intermed, Nunc) using wash buffer (Section Z). These plates remained stable at 4 °C and usable throughout the duration of the experiments.

The biotin attached to the BSA coating the assay plates was saturated with streptavidin. To saturate the biotin–BSA coated wells, 2 µg streptavidin/ ml streptavidin saturation buffer (Section Z) was incubated in each well for 3 h at room temperature. Each streptavidin molecule may theoretically bind up to four biotin molecules (Chaiet & Wolf, 1964).

To determine the success of the biotin–labelling of BSA and the coating of the microtiter plate, a sample plate from each batch of plates produced was tested. The plates were incubated with horseradish peroxidase–conjugated streptavidin in streptavidin saturation buffer at a concentration of 5 µg/ml for 3 h at room temperature. Following incubation, the plates were washed 10 times with wash buffer (Section

Z). For reliability and ease, the ortho–phenylenediamine dihydroxide (OPD) tablet, substrate buffer and stopping solution from a Kallestad™ Pathfinder ® Chlamydia Microplate kit (Sanofi Diagnostics Pasteur, Inc., Chaska, Montana) were used. The tablet was dissolved in 10 ml of substrate buffer and mixed by inversion just prior to use. To each well, 100 µl of this solution was added and incubated in the dark for 30 min. After incubation, 100 µl of stopping solution was added to each well. The development of an orangy–red colour indicated the presence of HRP and thus, biotin. The plates were read in an LP 400 Microplate reader. The readings significantly above the control wells indicated the successful biotin coupling and BSA–biotin coating of the wells.

With the particular plates used, blocking of non–specific binding of the europium–labelled probe was required. Each well was incubated at 45 °C overnight with 300 µl of Streptavidin saturation buffer (Section Z) with 20 mg/L of sheared herring sperm DNA and 1 % BSA. The plates were then washed ten times with the wash buffer (Section Z) and stored for future use at 4 °C.

W. Hybridization

The various probes constructed and used in the assay appear in Table 2. Two probes and the viral DNA were allowed to hybridize in preparation for capture on the collection plate. Clinical adenovirus isolates and their cultures (Table 3) and known amounts of HAd3 DNA were used in the assay. The virions were disrupted by repeated freezing at -70 °C and then boiling for five minutes. The sample was

Table 2. Probe construction.

Probe	Construction Method	Length NTs**	Number of Labels
Europium			
L157*	Aminolink 2™	30	1
E157	ligation with C25-Eu	30 + 25	1 - 25
E157*	PCR & strand deletion	54	1 - 7
E157	terminal transferase with Eu-dCTP	30 +	1 +
E157*	transamination	30	1 - 7
Biotin			
L477	Aminolink 2™	30	1
B477	biotin amidite	30	1
A477	ligation with C25-biotin	30 + 25	1 - 25
A477	terminal transferase b-dUTP	30 +	30 +
A477	transamination	30	6
A477*	unidirectional PCR with b-dUTP	43	1 - 8

* Not tested in assay.

** Nucleotides

Table 3. Clinical isolates of adenovirus and their sources.

Sample	Source	Culture	ElectronMicroscope
6787	feces	positive	negative
6597	auger suction	positive	–
7212	auger suction	positive	–
6672	feces	positive	negative
7137	feces	positive	negative
7110	auger suction	positive	–
6789	auger suction	positive	–
6959	nasopharyngeal swab	positive	–
7363	throat swab	positive	–
6986	feces	positive	negative

boiled for 10 minutes to denature the DNA and placed on ice and centrifuged.

The sample and the two probes were incubated in the presence of hybridization buffer (Section Z) at 55 °C for 1 hr: 100 µl disrupted sample or purified viral DNA was added to 100 µl hybridization solution in a 0.5 ml microfuge tube.

The europium probes tested were europium–labelled C25 ligated to E157 and Eu–dCTP terminal transferase labelled E157. Biotin probes used in the assay were aminolinked L477, biotin amidite B477, b–dUTP terminal transferase labelled A477, and biotin–labelled C25 ligated to A477.

X. Sandwich capture onto plate.

The hybridized DNA sandwich was bound to the plate via the interaction of the biotin–labelled probe with the streptavidin bound to the BSA–conjugated biotin. The hybridization mixture was incubated for 1 h at room temperature with collection buffer (Section Z) to permit the binding of the streptavidin to the biotin. After incubation, the plates were again washed ten times using the wash buffer (Section Z) using an Immuno Wash 8 plate washer .

Y. Time–resolved fluorimeter reading

Once the hybridization sandwich was ready, the plates were read in a 1232 DELFIA Fluorimeter (Wallac Oy, Turku, Finland). The instrument was configured to a Ratio format. Europium was excited at

340 nm and read at 613 nm and 1000 readings per second. The average counts of those readings was presented on screen and recorded. Background was evaluated for each plate by counting wells with no template DNA but with probes and buffers; five times above this background was arbitrarily considered to be positive.

Z. Buffers

Coating Buffer
0.9 % NaCl
50 mM K_2HPO_4, pH 9.0
0.05 % NaN_3

Collection Buffer
1 M NaCl
50 mM Tris-HCl, pH 7.75
0.2 % Tween-20
0.5 % BSA
0.05 % bovine globulin
0.05 % NaN_3

Dialysis buffer A
0.9 % NaCl
50 mM K_2HPO_4, pH 9.0
0.05 % NaN_3

Dialysis buffer B
5 mM NaH_2PO_4, pH 8.5

Dialysis buffer C
12 mM Tris-HCl, pH 7.6

Hybridization Buffer
500 mM NaCl
50 mM HEPES, pH 7.5
0.5 % Tween-20
100 µm EDTA.

NAP column buffer
20 mM HEPES, pH 7.5
0.9 % NaCl

PBS

137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4 \cdot 7H_2O$
1.4 mM KH_2PO_4

Polyacrylamide Gel Loading Buffer

95 % Formamide
0.1 % Bromophenol blue (w/v H_2O)
0.1 mM EDTA

SA–Saturation Buffer

50 mM Tris-HCl, pH 7.75
0.1 % Tween-20
0.5 % BSA
0.05 % NaN_3

SSC

3 M NaCl
0.3 M $Na_3citrate \cdot 2H_2O$, pH 7.0

Streptavidin saturation buffer

50 mM Tris-HCl, pH 7.75
0.1 % Tween-20
0.5 % BSA
0.05 % NaN_3

TBE

89 mM Tris-HCl, pH 8.0
89 mM H_3BO_3
2 mM EDTA, pH 8.0.

TBS

20 mM Tris-HCl, pH 7.6
500 mM NaCl

TE

10mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

TPBS

0.05 % Tween-20 in PBS

Wash Buffer

10 mM Tris-HCl, pH 7.75
0.9 % NaCl
0.05 % Tween-20
0.05 % NaN_3
0.1 % BSA

RESULTS

A. Sensitivity using PCR

The specificity of the probes selected from the hexon coding region of the HAd3 genome were tested using the polymerase chain reaction (PCR). A 320 base pair segment of the gene was amplified using the primers E157 and A477. Various levels of template DNA were subjected to PCR using oligonucleotides E157 and A477 (Figure 8). Template titration was evaluated on a 1 % agarose gel by electrophoresis at 150 V for 45 min. Amplification was seen using 250 pmol, 25 pmol, 2.5 pmol, and 250 fmol of template HAd3 DNA. No band was seen using 25 fmol of template HAd3 DNA.

B. Clinical adenovirus isolates recognized by PCR

Adenovirus positive isolates from the virology laboratory of the division of Microbiology and Public Health, University of Alberta Hospitals, were subjected to PCR (Figure 9). Oligonucleotide primers E157 and B477 were used to test their ability to recognize adenovirus isolates. Cell culture positive specimens 6789, 6959, and 7363, in lanes 8, 9, and 10, respectively, produced the expected 320 base pair product. Lane 12 is a positive control with 2.5 pmol of HAd3 DNA and produced an identical PCR product.

The polymerase chain reaction was used to ensure that transaminated, europium–labelled oligonucleotide probes could still

Figure 8. Agarose gel electrophoresis of PCR titration of HAd3 DNA using oligonucleotides E157 and B477. Lane 1, 1 Kb marker (Gibco, BRL); lane 2, 250 pmol HAd3 DNA; lane 3, 25 pmol HAd3 DNA; lane 4, 2.5 HAd3 DNA; lane 5, 250 fmol HAd3 DNA; lane 6, 25 fmol HAd3 DNA; lane 7, negative control.

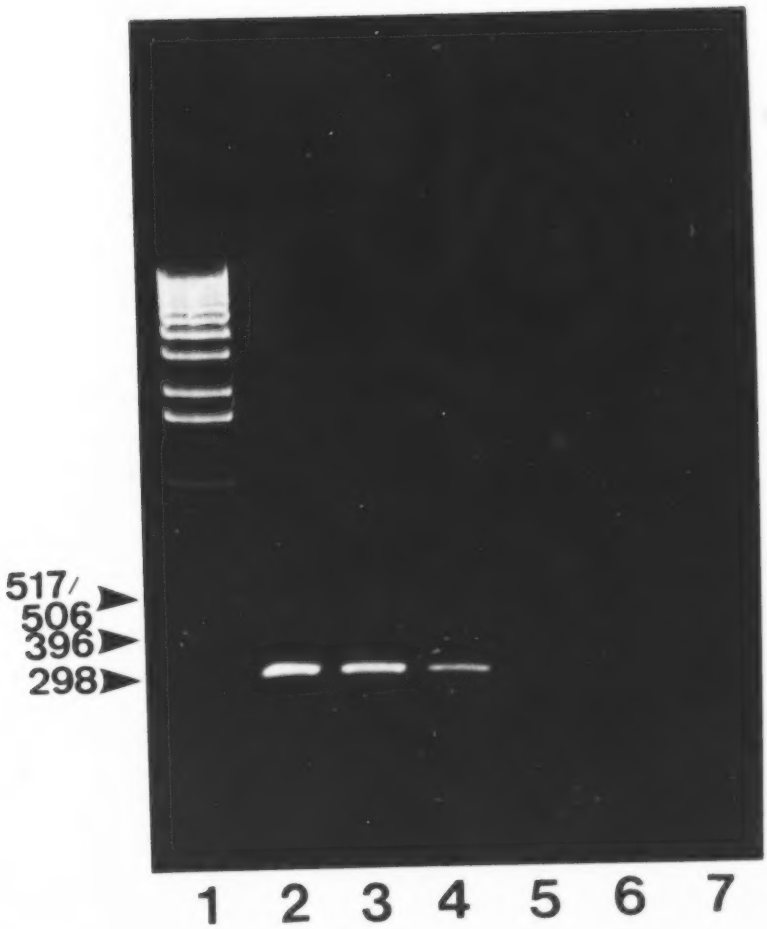
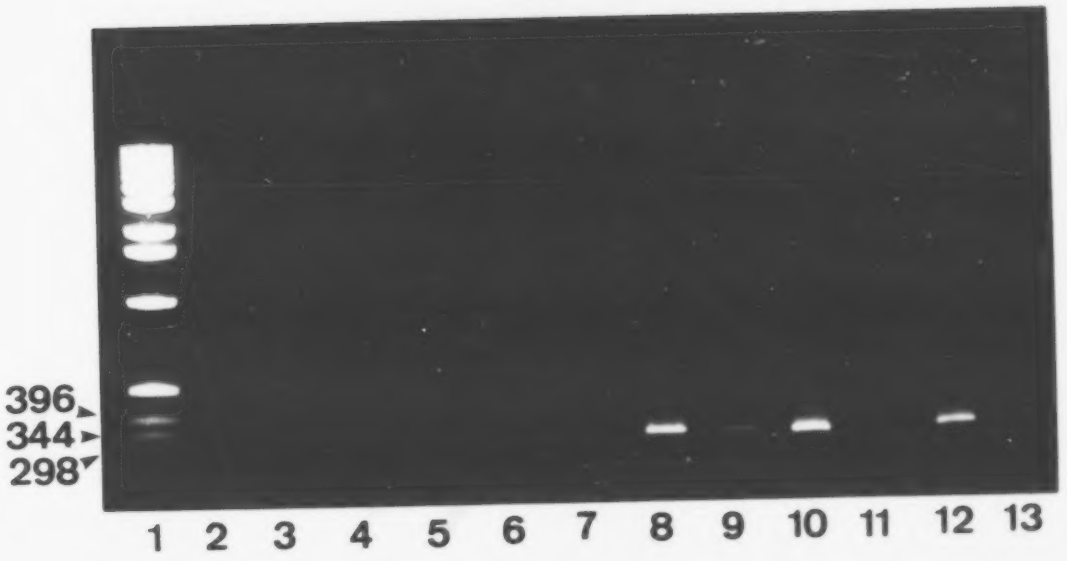

517/
506
396
298
1 2 3 4 5 6 7

Figure 9. Agarose gel electrophoresis of PCR using E157 and A477 of hospital isolates of adenovirus. Lane 1, 1 Kb marker (Gibco, BRL); lane 2, 6787; lane 3, 6597; lane 4, 7212; lane 5, 6672; lane 6, 7137; lane 7, 7110; lane 8, 6789; lane 9, 6959; lane 10, 7363; lane 11, 6986, lane 12, positive control, 2.5 pmol HAd3 DNA; lane 13, negative control.


396
344
298
1 2 3 4 5 6 7 8 9 10 11 12 13

recognize HAd3 DNA (Figure 10). The expected 320 base pair products were not seen using an annealing temperature of 55 °C. Using a lower annealing temperature of 45 °C only lanes 8, 9, and 10 which contained clinical specimens 6789, 6959, and 7363, respectively, produced the expected PCR product on 1 % agarose gel (Figure 11). Other unexpected bands were also seen. The primers were known to only amplify a 320 base pair segment (see Materials & Methods section).

C. PCR used to create probes

Oligonucleotide probe E157 (transaminated and europium labelled) and primer, B210 (biotin–labelled) were used in polymerase chain reaction to create a 54 base probe. PCR products were examined on a 2 % agarose gel (Figure 12). The PCR product was denatured and streptavidin was added to bind the biotin and removed by phenol chloroform. The double–stranded PCR product and the single–stranded purified europium–labelled probe were blotted onto nylon membrane Hybond™–N nylon membrane to demonstrate the removal of the biotin–labelled probe (Figure 13). These samples were also examined on 20 % polyacrylamide gel to demonstrate the presence of DNA after phenol/chloroform extraction of the biotin–labelled strand (Figure 14).

D. Oligonucleotide probe inspection

The probes created by aminolinking to biotin, synthesizing with biotin amidite, terminal transferase labelling with b-dUTP, ligation

Figure 10. Agarose gel of hospital isolates of adenovirus and transaminated, europium–labelled E157 and A477, annealing temperature 55 °C. Lane 1, 1 Kb marker (Gibco, BRL); lane 2, 6787; lane 3, 6597; lane 4, 7212; lane 5, 6672; lane 6, 7137; lane 7, 7110; lane 8, 6789; lane 9, 6959; lane 10, 7363; lane 11, 6986; lane 12 positive control, 2.5 pmol HAd3 DNA; lane 13 negative control.

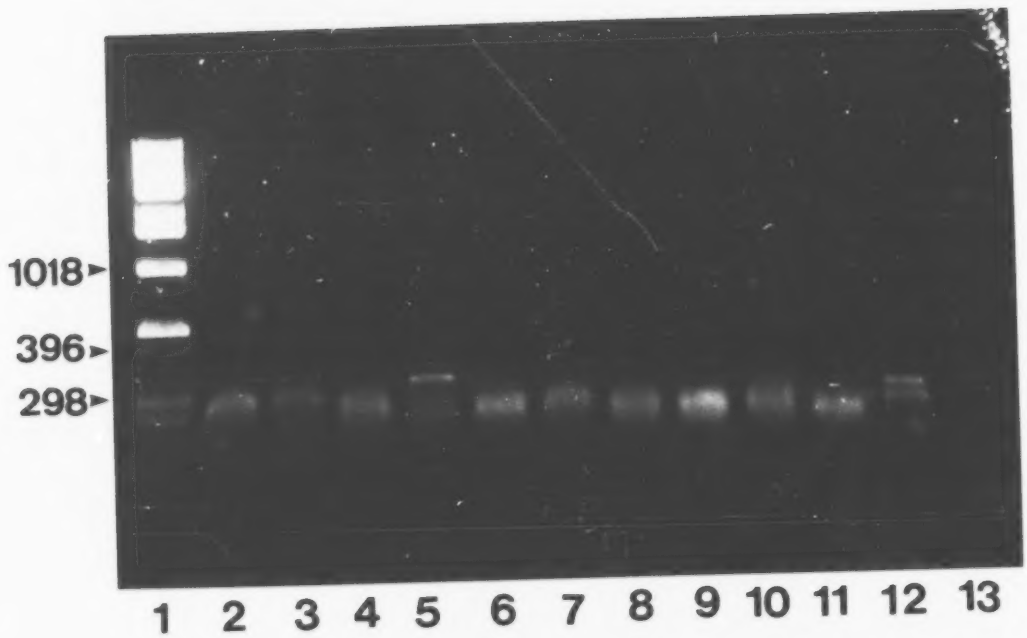

1018
396
298
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 11. Agarose gel of hospital isolates of adenovirus and transaminated europium–labelled E157 and A477, annealing temperature 45 °C. Lane 1, 1 Kb marker (Gibco, BRL); lane 2, 6787; lane 3, 6597; lane 4, 7212; lane 5, 6672; lane 6, 7137; lane 7, 7110; lane 8, 6789; lane 9, 6959; lane 10, 7363; lane 11, 6986, lane 12, positive control, 2.5 pmol HAd3 DNA, lane 13 negative control.

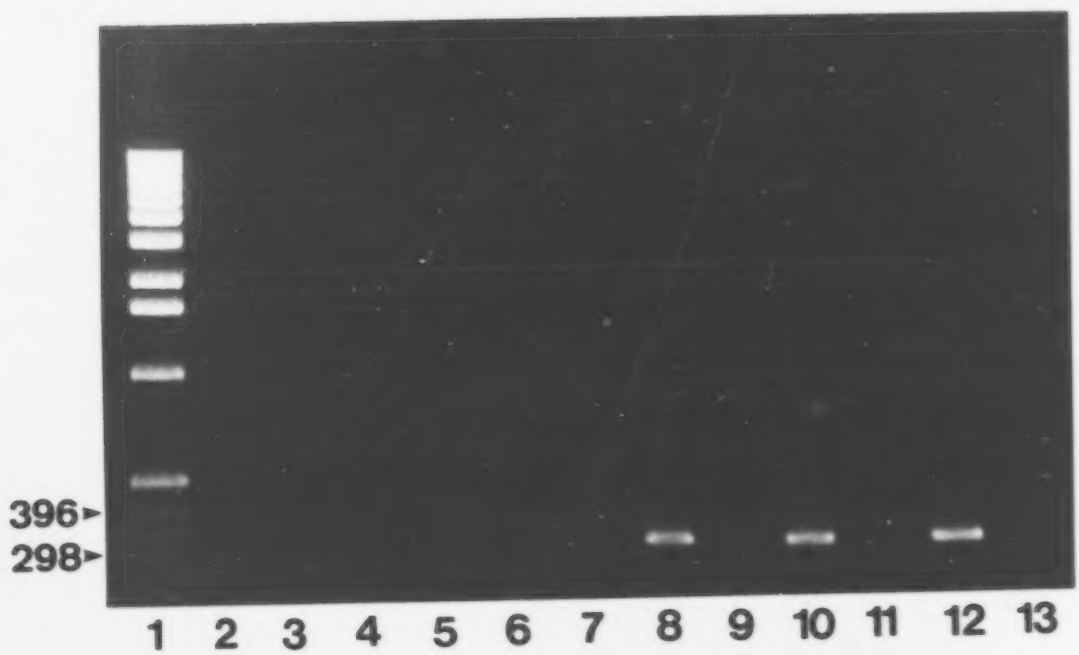

396
298
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 12. 54 base–pair PCR product of europium–labelled E157 and B210. Lane 1 is a 1 kb molecular weight marker, lane 2 is the 54 base–pair PCR product, and lane 3 is a negative control.

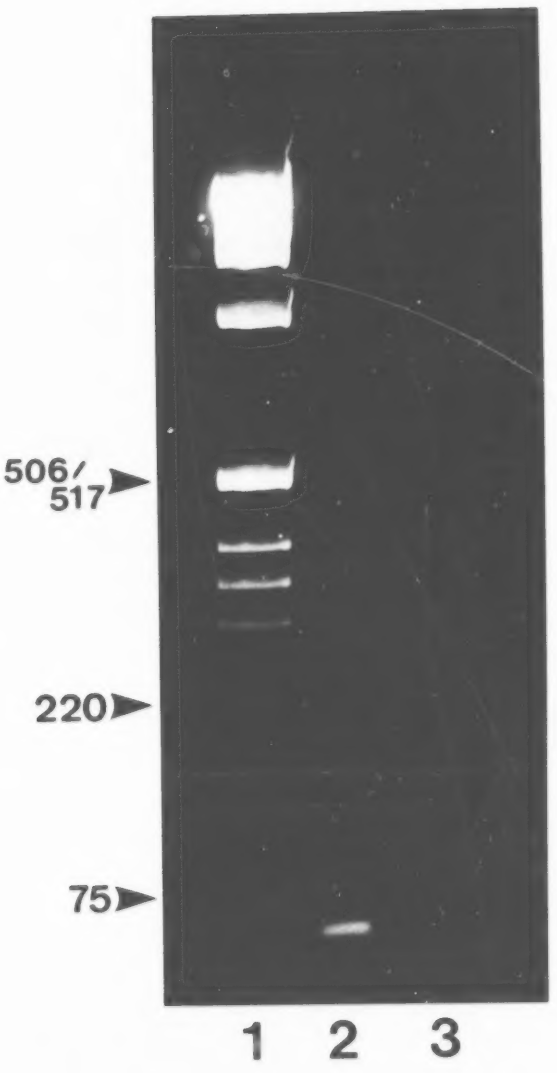

506/
517
220
75
1
2
3

Figure 13. Dot blot biotin–labelled strand removal by phenol chlorofrom extraction. Well 1, double stranded PCR product with biotin–labelled strand and europium–labelled strand. Well 2, europium–labelled probe purification after two phenol/chloroform/ isoamyl alcohol extractions. Well 3, purified europium–labelled strand after 4 phenol/chloroform/isoamyl alcohol extractions.

1
2
3

Figure 14. 20 % PAG of biotin–labelled strand removal by phenol chlorofrom extraction. Lane 1, double stranded PCR product with biotin–labelled strand and europium–labelled strand. Lane 2, europium–labelled probe purification after two phenol/chloroform/ isoamyl alcohol extractions. Lane 3, purified europium–labelled strand after 4 phenol/chloroform/isoamyl alcohol extractions.

1 2 3

with biotin–labelled C25, and transaminated and labelled with biotin were inspected by blotting directly onto nylon membrane (Figure 15). Biotin–labelling success was qualitatively examined using dot blotting of the biotin–labelled probes onto Hybond™–N nylon membrane. The membrane was incubated with HRP–conjugated streptavidin. The development of a red colour on the membrane crosslinked to the desired DNA indicated the successful labelling of that DNA with biotin. The various europium– and biotin–labelled probes were examined on 20 % polyacrylamide (29:1 acrylamide:bis–acrylamide) gels (Figures 16 and 17).

E. Biotin Labelling of BSA

Hierholtzer *et al.*, (1993) recommended the use of 0.12 ml of 1 mg/ml biotinamidocaproate N–hydroxysuccinimide ester in DMSO. However, they did not suggest a volume of 1 mg/ml BSA with which to mix the biotin. To determine what ratio of BSA to ester yielded the most efficient and economical labeled BSA, 1 ml of 1 mg/ml BSA in Dialysis buffer C was combined with 0.12, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, and 1ml of 1 mg/ml biotin ester in DMSO.

The resulting products were blotted onto nitrocellulose membrane and reacted with HRP–conjugated streptavidin (Figure 18).

F. Sensitivity

Various europium– and biotin–labelled probes were used to detect standard amounts of HAd3 DNA and clinical isolates. The

Figure 15. Nylon blotting of biotin–labelled probes. Well 1 is B210, well 2 is B477, well 3 is bC25, lane 4 is bC25 ligated to A477, and well 5 is unidirectional PCR using b-dUTP.

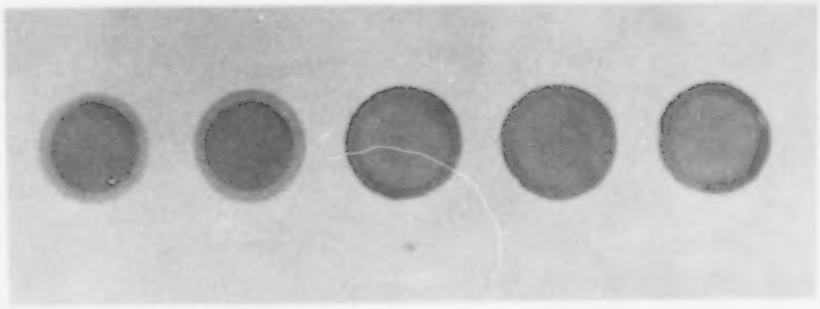

1
2
3
4
5

Figure 16. 20 % PAG of some probes. Lane 1 is C25, lane 2, a 19 base oligonucleotide marker, lane 3, unlabelled E157, lane 5, E157 terminal transferased with europium–labelled dCTP, and lane 6, E157 ligated to transaminated, europium–labelled C25.

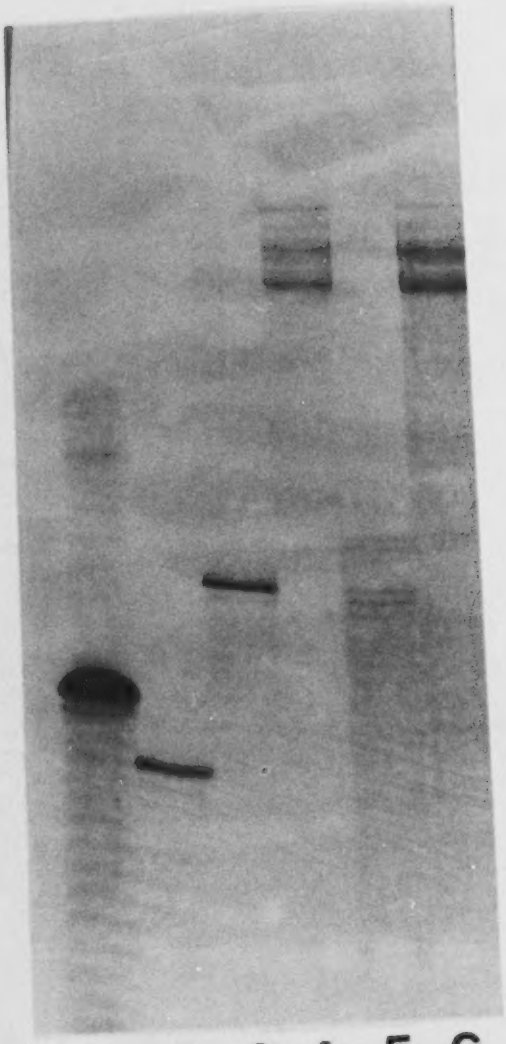

1 2 3 4 5 6

Figure 17. Some labelled probes on 20 % acrylamide gel. Lane 1 is E157 terminal transferased with europium–labelled dCTP, lane 2 is E157 ligated to europium–labelled C25, lane 3 is the oligonucleotide C25, and lane 4 is the 1 kb marker.

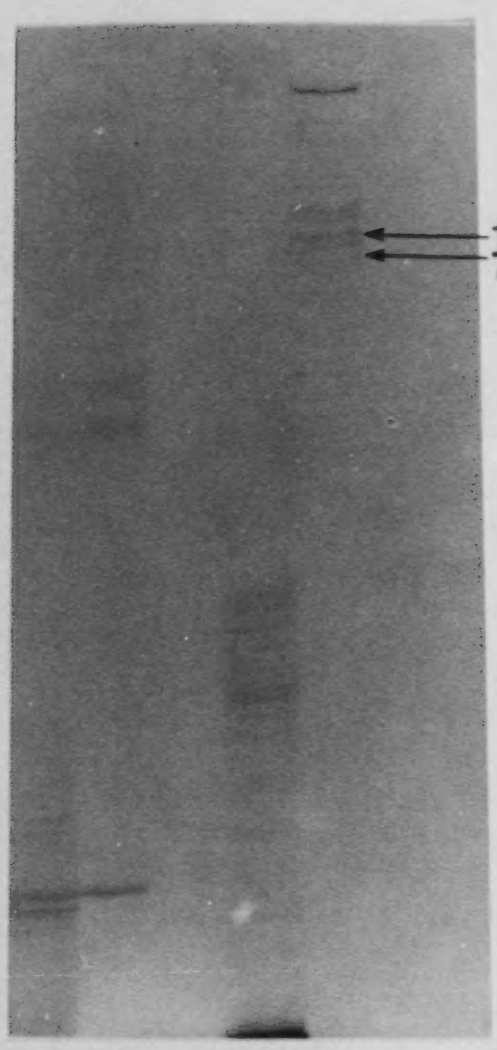
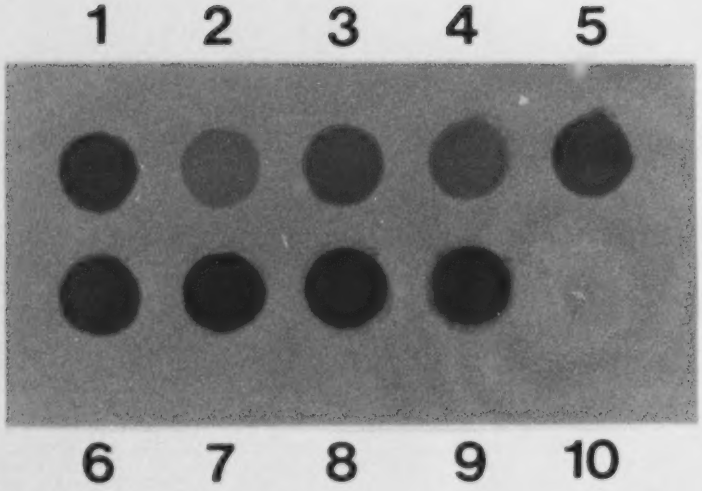

154
75
1 2 3 4

Figure 18. Dot blotting of biotin–labelled BSA. Well 1 is 0.12 ml biotin in DMSO, wells 2-9 contain 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, and 1ml of 1 mg/ml biotin ester in DMSO respectively; well 10 is 0.1 mg unlabelled BSA.


1 2 3 4 5
6 7 8 9 10

results were compared to PCR results to determine the sensitivity and specificity of each combination of probes. Figure 9 identifies clinical isolates 6789, 6959, and 7363 as HAd3 positive. Because these primers did not identify HAd3 sequences in the other clinical samples used, they were not expected to give positive hybridization results. Therefore, these negative isolates were used to determine background values; five times the background level was arbitrarily accepted as the cutoff for a positive result. Table 4 summarizes the counts per μg of each of the labelled probes. Figure 19 compares the sensitivity of the E157 terminal transferase and E157 C25 ligated europium–labelled probes with B477 used to detect standard amounts of HAd3 DNA. Using 50 ng/ml of B477 and the europium–labelled probe constructed using terminal transferase, 25 fmol (575 ng) of HAd3 DNA was detected. Using 50 ng/ml of B477 and the europium probe constructed by ligating europium–labelled C25 1 pmol (23 μg) was detected. Table 5 summarizes the average background counts, value required for a positive, and the value above the positive cutoff of the HAd3 positive isolates.

Table 4. Europium–labelled methods and counts per pmol.

Method of labelling	Counts per pmol DNA
standard Eu	10,417
Aminolink 2™	598
ligation with C25-Eu	433,637
PCR & strand deletion	1096
Terminal transferase with Eu-dCTP	1,407,840
transamination	4,814,880
background	398

Figure 19. Comparison of sensitivity of the europium–labelled probes with B477. ■ : E157 terminal transferased with Eu–dCTP. ▲ : E157 ligated to Eu–C25.

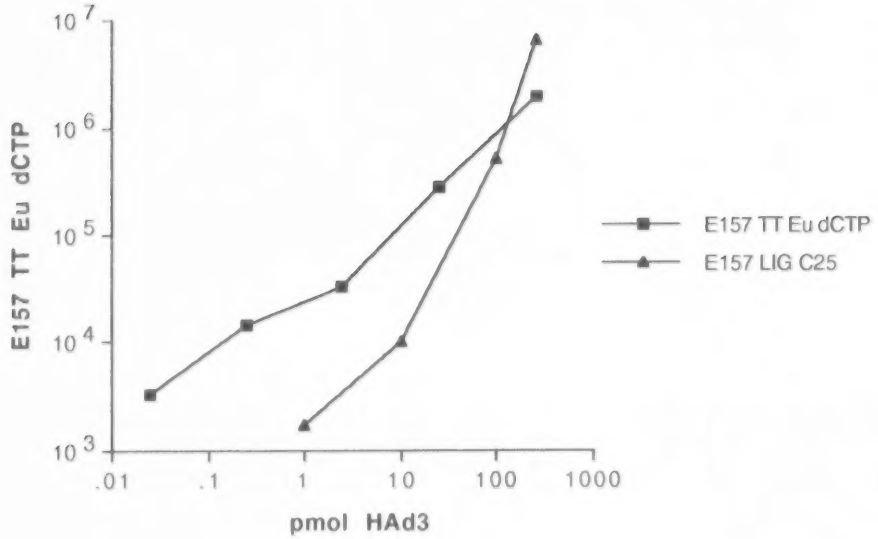

E157 TT Eu dCTP
10 7
10 6
10 5
10 4
10 3
.01
.1
1
10
100
1000
pmol HAd3
E157 TT Eu dCTP
E157 LIG C25

Table 5. Clinical counts of probes. Background values are averaged. Positive is considered to be arbitrarily 5 times above background. Actual times counts above background presented.

Probes	Background	Positive*	Times Above Positive
E157 lig EuC25 bL477	222	1100	65 17 35 438
E157 lig EuC25 B477	266	1330	245 33 156 460
E157 lig EuC25 A477 lig bC25	225	1120	343 55 444 555
E157 lig EuC25 A477 TT	253	1270	622 46 310 455
E157 lig EuC25 A477 TA	263	1320	334 50 92 31

* 5 times above background

Table 5 Continued. Clinical counts of probes

Probes	Background	Positive*	Times Above Positive
E157 TT	23,309	116,540	65
bL477			37
			69
			34
E157 TT	27,057	135,290	79
B477			31
			39
			46
E157 TT	24,358	121,790	19
A477 TT			13
			70
			7.9
E157 TT	17,413	87,070	27
A477 lig bC25			18
			26
			20
E157 TT	12,849	64,250	7.2
A477 TA			9.3
			11
			11

*5 times above background

DISCUSSION

Of the many choices of diagnostic techniques, nucleic acid hybridization methodologies have become more important to clinical as well as research laboratories. The main advantage of nucleic acid hybridization is its absolute specificity. Because they directly identify genetic material, nucleic acid probes do not need secondary indicators, such as antigens. DNA probes can discriminate among organisms in mixed cultures, detect nonviable organisms, and uncover differences in nucleotide sequences characteristic of genetic mutations or alterations.

In DNA hybridization studies, radioactive labels are the most common method of detection. However, enzymatic, colourimetric, chemiluminescent, biotin–streptavidin interactions, and now, time–resolved fluorimetry methods, have gained in popularity as improved methods often approach the sensitivity of radiolabels required for widespread use (Wolcott, 1992).

Europium is a fluorescent label which offers the sensitivity required for diagnostic assays without the hazards of radioactive labels. Due to the unique time–resolved fluorescence characteristics of europium and other lanthanides, the fluorescence of other compounds, such as bovine serum albumin, does not create background interference. This ability to discriminate between impeding and diagnostic fluorescence makes europium an ideal candidate for fluorimetric assays. This research has tried to capitalize on the advantageous characteristics of time–resolved fluorescence to

develop a hybridization assay which can be broadly used in clinical and research laboratories.

However, because minute amounts of europium are detectable, this system is highly susceptible to carry–over of the hybridized DNA or the labelled probes themselves. Preventing contamination by extraneous labelled probe is important in ensuring accurate detection of target DNA. This was accomplished in this study by careful manipulation of labelled DNA, use of dedicated pipettes and disposable equipment, and stringent plate washing.

In addition, the use of polystyrene 96–well plates posed a problem in the design of the assay. Although convenient and inexpensive, commercially available 96–well plates have a negative charge which attracts and binds the trivalent cation, europium. This produces a high background signal and increases the incidence of falsely positive results. It was possible to purchase specially designed low–binding plates (Nunc); however, this was inconsistent with the principle of this thesis: to develop a use for this technology with optimal efficiency and flexibility of application. Instead of designing the assay around specific materials such as treated plates (Dahlén *et al.*, 1991b), the available plates were modified to suit the parameters of the assay. Blocking was best accomplished with the solution recommended by Lopez *et al.* (1993). Other researchers do not report blocking the plates (Dahlén *et al.*, 1993, Hierholtzer *et al.*, 1993, Iitia *et al.*, 1992a, Iitia et al., 1992b, Iitia *et al.*, 1992c) or report solutions which were not found to be suitable (Dahlén, 1987, Dahlén et al. 1987) as background readings disguised true hybridizations.

PCR is one of the more sensitive methods in use today to detect given DNA sequences. The titration of HAd3 DNA by PCR revealed that 250 fmol of template was detectable using 40 pmol each of oligonucleotides E157 and B477 and agarose gel electrophoresis. However, 25 fmol of template could not be visualized on agarose after 30 cycles of amplification. Also, neither 250 pmol, nor 25 pmol of HAd3 template yielded significantly more product than 2.5 pmol. Under optimal PCR conditions, using 40 pmol of each primer, 250 fmol of template could be detected by PCR but 2.5 pmol of template gave results with more certainty.

Using PCR, three of the ten adenovirus samples obtained from University of Alberta Hospitals Virology laboratory were positive using 40 pmol each of the oligonucleotides, E157 and B477. Two of the isolates, 6789 and 7363, were of equal intensity as the positive control of 2.5 pmol of HAd3 DNA on the agarose gel. The 6959 isolate was of a lesser intensity. Since these isolates were crudely prepared by freeze boiling and not phenol/chloroform extracted and ethanol precipitated, one cannot comment on the amount of DNA originally added. As well, since only one region of the HAd3 was investigated, one cannot comment on the true identity of these isolates; the point was to use a specific sequence to identify a specific region of HAd3 genome, not to investigate adenovirus types. However, because the oligonucleotides were selected from published sequences for HAd3 (Sussenbach, 1984), they were also not expected to recognize other adenovirus types. Therefore, these oligonucleotides identified the desired sequence in three of the ten clinical adenovirus isolates.

The effect of different concentrations of probes on sensitivity was also investigated. Using PCR to first amplify their target DNA, Iitia *et al.*, (1992a) reported that 2.0 ng (88 fmol) of europium–labelled probe (30 europium molecules per probe) and 0.5 ng (50 fmol) of biotin–labelled probe (one biotin molecule per probe) was optimal for detection of HTLV target DNA. Hierholtzer *et al.*, (1993) recommended 30 ng each of the europium–labelled probe (20 europium molecules per molecule) and the biotin–labelled probe (one molecule of biotin per molecule) after amplification of adenovirus template using PCR. Because the target DNA was not amplified in this thesis, higher levels (50 ng/ml) of probes were needed. PCR products are generally detected using agarose gel electrophoresis and ethidium bromide fluorescent staining. The lowest levels detectable by this method are 1 - 5 ng (Sambrook *et al.*, 1989). The sensitivity of europium is such that 100 fmol of europium is detectable. Theoretically, assuming each probe has 25 europium molecules, 4 fmol or 40 pg of labelled DNA can be detected using a time–resolved fluorimeter. Hurskainen *et al.* (1989) reported that europium–labelled lambda DNA probe could detect 10 pg (0.3 amol). Thus, lower levels are detectable by this method using 500 ng/ml europium–labelled probe, than visual inspection using agarose gel electrophoresis.

The titration assay using HAd3 DNA revealed that, the E157 probe labelled by terminal transferase was more sensitive than the ligated probe. However this was expected because of the higher counts per pmol probe. In general, with more europium labels in the probe, the more sensitive the assay; however, too many europium groups linked directly to the probe resulted in a decreased melting temperature

and subsequent decreased specificity (Figure 11). Europium or biotin labels attached to modified amino groups of deoxycytidine residues result in fewer hydrogen bonds available for base pairing (Hurskainen *et al.*, 1991) thus weakening the attraction of the two complementary DNA strands. Using a higher europium–probe concentration could compensate for the decreased specificity of a transaminated and europium–labelled E157 (Hurskainen *et al.*, 1991) but because of the decrease in specificity this was not investigated. It was felt that with other probes available, the possibility of non–specific binding as seen with PCR (Figure 11) did not warrant further use of this probe. As well, too high a europium probe concentration would likely result in higher background counts. Biotin labelling did not approach levels reported to inhibit base pairing (Langer *et al.*, 1981) and a single biotin attached to the end of the probe is sufficient to capture the hybridized DNA in this study as well as in the literature (Dahlén *et al.*, 1991a, Dahlén *et al.*, 1991b, Dahlén *et al.*, 1993, Hierholtzer *et al.*, 1993, Iitia *et al.*, 1991, Iitia *et al.*, 1992b Iitia *et al.*, 1992c). Too high a biotin probe concentration was reported to compete with streptavidin binding sites on the well (Iitia *et al.*, 1992a); however, this effect was not seen in this research project.

A variety of labelling procedures have been presented in the literature. Methods that have been suggested include synthesis of oligonucleotide probes using europium–labelled deoxycytidine (Dahlén *et al.*, 1994), europium and biotin labelling of amino–modified deoxycytidine (Bush *et al.*, 1992; Dahlén *et al.*, 1993; Dahlén *et al.*, 1991a; Dahlén *et al.*, 1991b; Hierholtzer *et al.*, 1993; Iitiä *et al.*, 1992a; Iitiä *et al.*, 1992b; Iitiä *et al.*, 1992c), amino–modified and 4,7-bis (chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)

–labeled oligonucleotides detected with europium–labelled anti–BCPDA antibodies (Chan *et al.*, 1993). Several different groups have utilized transamination of oligonucleotides with subsequent europium and biotin labelling (Dahlén *et al.* 1988; Gillam & Tener 1986; Hurskainen *et al.*, 1991; Viscidi *et al.*, 1986). Europium–labelled antibodies to double stranded DNA (Diamandis & Christopoulos, 1990) or other substances attached to the DNA (Lopez *et al.*, 1993; Diamandis & Christopoulos, 1990; Syvänen *et al.*, 1986) have seen some use. Less popular is the incorporation of biotin–labelled deoxyuracil triphosphate (Coultée *et al.* 1991). The use of europium–labelled streptavidin to detect biotin–labelled nucleic acid has been explored by different groups (Christopoulos *et al.*, 1991; Dahlén, 1987; Dahlén *et al.*, 1987; Prat *et al.*, 1991; Suonpaa *et al.*, 1992). Convenience, ease of use, and cost of these methods vary greatly and will thus affect which method is adopted by a particular laboratory. In addition to these reported procedures, I have suggested linking biotin– and europium–labelled polycytosine tails to probes; using PCR with biotinylated dUTP or europium– and biotin–labelled primers to create probes; and tailing probes with europium– or biotin–labelled nucleotides.

As the assay was designed to be simple, universally applicable, and well suited for a kit format, a number of europium and biotin labelling methods were tried and evaluated. The labelling methods most easily adopted by a laboratory interested in using this assay would have to be simple, easy to perform, and should not require extraordinary chemicals or equipment.

Of the methods mentioned, the easiest were those which involved labelling during synthesis, that is, those with the labelled

phosphoramidites incorporated into the synthesis program. The synthesis products were consistent in that the number of labelled bases was predicable, also, they were simple to purify using OPC™. However, the expense of such convenience is considerable as labelled phosphoramidites are costly.

Using Aminolink 2™ to label the oligonucleotides was also fairly simple, requiring only a few small additional steps after synthesis. Again, synthesis products were fairly predictable assuming 100 % labelling. Purification using OPC™ was also possible. This method was less expensive than direct incorporation during synthesis and affordable for a laboratory considering adopting this method.

The europium–labelled probe, L157 synthesized with an Aminolink 2™ at the 5′ end, did not result in acceptable levels of labelling. Bush *et al.*, (1992) report using the Aminolink 2™ to incorporate one europium chelator into the probe; however, they used a nested hybridization after amplification of the target by PCR. It is uncertain whether the synthesis of the oligonucleotide or labelling of the amino group was unsuccessful. However, the mere fact that only one europium molecule is attached per probe suggests that this would not generate enough signal per probe to be useful. Hurskainen *et al.*, (1991) report that labelling 4 to 8 % of total nucleotides with europium is optimal. Therefore, this method of labelling was not investigated further in the assay.

Transamination of oligonucleotides was inexpensive and simple, however far less convenient than synthesizing directly. In addition, the success of labelling was not predictable and difficult to determine. Evidence also suggests that the transaminated, labelled

probe is less specific than desirable. PCR using A477 and transaminated europium–labelled E157 was not permissive using an annealing temperature of 55 °C, whereas non–transaminated E157 produced the expected 320 base pair product at 55 °C (Figures 9 and 8, respectively). The transaminated europium–labelled E157 did produce the expected 320 base–pair product at 45 °C (Figure 11). Hurskainen *et al.*, (1991) report a 0.7 °C decrease in melting temperature (Tm) per percent of europium labelled. However, additional bands were seen on the agarose gel, indicating non–specific binding at the lower annealing temperature and thus the specificity of transaminated, europium–labelled oligonucleotide was decreased. In contrast, Hurskainen *et al.*, (1989) reported that, although 10 % europium–labelling results in decreased sensitivity and Tm, they did not observe a decrease in specificity. This evidence suggests that transaminated, europium–labelled probes may have some use in other assays.

To regain specificity of the transaminated probes, PCR was used to extend the probe with unlabelled flanking sequences (Figure 11). The complementary strand was removed by phenol/chloroform extraction. While Figures 14 and 15 indicate that the amplification product of europium–labelled E157 and B210 has been purified and the biotinylated strand extracted, it is still a possibility that not all of the biotinylated strand may have been removed. The significance of this is that if the biotin–labelled strand should remain with the probe, it could compete with target HAd3 DNA for europium–labelled probe binding. These short oligonucleotides anneal more favourably than the longer DNA. As well, since this strand is biotin–labelled, it is capable of binding to the biotin capture mechanism and causing falsely positive

results. Considering these risks and the availability of europium labelled nucleotides (Dahlén *et al.*, 1994), as well as the convenience of synthesizing probes directly, this method of creating probes is not favoured. As well, the evaluation of the final, purified europium–labelled probe revealed very low counts per μg of probe. Since directly transaminated and labelled E157 yielded high counts, the decrease was thought to be brought about by the phenol/chloroform/isoamyl extraction. In addition to the concern of residual biotin–labelled strand and the possibility of false positives, this finding dismisses the possibility of using this method to create europium–labelled probes.

Transaminated and europium– or biotin–labelled non–base–pairing polycytosine was ligated to the unlabelled probe sequences E157 and A477. The preparation time of the probe was greatly increased and the waste of unincorporated segments is considerable as the acceptor terminus (3′–OH) must be supplied in excess of the donor terminus (5′–P) to favour ligation rather than concatamerization of the donor. Visualization of ligated products is difficult and the extent of ligation is unpredictable and confusing if smaller fragments are in the ligation mixture. Transamination and europium–labelling of the 25 mer cytosine oligonucleotide and subsequent ligation to E157 yielded acceptable counts per μg of probe.

Probes were also 3′ tailed with terminal transferase using labelled nucleotides. Additional expense was nominal and preparation time was slightly longer than with direct synthesis, however, the number of nucleotides incorporated was variable and difficult to predict (the enzymatic activity is characterized by the range of nucleotides incorporated) or visualize. Using terminal transferase and

europium–labelled dCTP was successful, with high counts per μg probe.

Langer *et al.*, (1981) reported that lightly labelled biotin probes (2 %) have essentially the same melting temperature as their unlabelled counterparts and a segment with 25 % of its bases labelled only produced a decrease in Tm of 5 °C. However, because the biotin – avidin interaction has one of the highest binding constants ($K_{dis} = 10^{15}$) known (Green, 1975) and because the literature suggests that, in using biotin–based capture, one biotin group attached to the capturing probe was considered to be sufficient (Dahlén *et al.*, 1991a, Dahlén *et al.*, 1991b, Dahlén *et al.*, 1993, Hierholtzer *et al.*, 1993, Iitia *et al.*, 1991, Iitia *et al.*, 1992b Iitia *et al.*, 1992c) the transaminated, biotin–labelled A477 and A477 ligated to transaminated, biotin–labelled C25 were not expected to produce superior results. Standard amounts of DNA were captured roughly equally by all biotin–labelled strands in each combination of oligonucleotides.

Data from the blotted biotinylated BSA suggest that the amount of biotin attached to the BSA can reach saturation. Because wells 1, 5, and 6 (0.12, 0.8, and 0.16 ml respectively) are only slightly lighter than wells 7, 8, and 9 (0.32, 0.64, and 1.0 ml respectively), economics dictated that sub–saturation levels of biotin ester were sufficient to coat the plates. Hierholzer *et al.* (1993) had success with 0.12 ml; 0.08 ml was found to be comparable to their labelling procedure and was used for all BSA labelling reactions.

The primary limitation and disadvantage of the design of this assay is the working volume. The wells of the plates are only 300 μl in volume and because buffers are added to assist in hybridization of the

labelled oligonucleotides to the target DNA and then the capture of the hybridized sandwiched DNA, the sample volume is necessarily decreased. This provides strong support for the use of PCR to amplify the target DNA to concentrations which are conducive to the small volumes of the assay wells.

Others who used PCR to first amplify the target DNA were able to demonstrate the PCR product on agarose gel (Bush *et al.*, 1992, Chan *et al.*, 1993, Christopoulos *et al.*, 1991, Hierholtzer *et al.*, 1993) They went on to use time–resolved fluorimetry to make the final diagnosis. PCR has proved to be able to make accurate detection and diagnosis of target DNA samples (Ehrlich & Greenberg, 1994). For these authors (Bush *et al.*, 1992, Chan *et al.*, 1993, Christopoulos *et al.*, 1991, Hierholtzer *et al.*, 1993) their subsequent step was redundant as they already had a positive diagnosis. Since it was seen that amplification of 25 fmol of standard HAd3 DNA could not be seen on agarose, and since time–resolved fluorimetry theoretically can detect 4 fmol, there is no logical reason to increase the sensitivity of PCR. Other authors claim to use time–resolved fluorimetry quantitatively (Chan *et al.*, 1993, Christopoulos *et al.* 1991, Hierholtzer *et al.*, 1993, Syvänen *et al.*, 1986). It has been my experience that this is not possible because of the nature of europium fluorescence. Europium has low quantum yield, and thus not all the molecules will fluoresce predictably. Because of the long–lived fluorescence, a good number of quantitatively useful photons will have decayed after time–delayed readings. In addition, in attempting to prepare standard curves using standard europium molecules it was difficult to achieve linear or consistent relationships between dilutions and counts. This could be due to binding of the

trivalent cation to the pipette tips or tubes. As well, micro environments at low concentrations may play an important role as fluorescence is enhanced by lower pH and neighboring molecules. In addition, many of the labelling techniques previously described in the literature and suggested here offer an unpredictable success of labelling. This makes calculation of target DNA difficult.

The method developed in this study can theoretically detect 4 fmol (40 pg) of DNA labelled with 25 europium molecules. In practice 50 ng/ml E157 terminal transferase labelled with Eu–dCTP was able to detect 25 fmol of purified HAd3 DNA; the E157 ligated to transaminated, europium–labelled C25 was able to detect 1 pmol (23 μg) of HAd3 purified DNA. These levels are lower than those reported by Hurskainen *et al.*, (1989) however, they used higher probe concentrations because of beneficial probe kinetics and efficiency at this high concentration. The level of sensitivity achieved in this project is comparable with PCR. The clinical cultures tested were centrifuged to attempt to concentrate the cellular debris containing virions. However, depending on the original isolate, this concentration may not be possible.

It is unfortunate that a more extensive examination of a range of clinical isolates to further optimize this technique could not be undertaken. Because of time constraints and the paucity of clinical adenovirus isolates, assessment of this technique as a screening assay was not possible. However, the point of this project was not to develop a test for adenovirus but rather to use adenovirus as a model around which to base a flexible and sensitive DNA detection assay.

There are many research laboratories actively developing specific assays for particular microorganisms. Clinical laboratories purchase these kits and specific equipment required to use them. Because it is more profitable to market one test for a specific application and another assay as a different kit, little research has been applied to improving the flexibility of an assay. However, this flexibility is needed as microorganisms are constantly changing in the face of treatments and antigenically and genetically variable microorganisms begin to avoid detection by diagnostic kits. If a DNA hybridization kit is supplied with a specific labelled sequence, the kit could very well become irrelevant with the genetic evolution of the specific microbe. While this may be profitable for the company producing the kits, it is frustrating and a hindrance to the diagnostic laboratory. Research laboratories will often develop their own detection kits in basic research. Often the assays are cumbersome, time consuming, and involve radioactive labels. Some groups are exploring non–radioactive labels such as digoxigenin and biotin–streptavidin. While these techniques do not require the purchase of kits and equipment, they still are often not as sensitive as radioactive labels.

Some of the labelling techniques explored in this project could easily be accomplished by both clinical and research laboratories. As well, many universities, research facilities, and molecular supply companies offer inexpensive and rapid delivery of specific oligonucleotides. If a specific sequence is needed to investigate a new or related microorganism, or a characteristic genetic disease locus, this may be accomplished by simply ordering or synthesizing the probe needed. As well, because the plates may be prepared and stored in the

laboratory conducting the assay and the sequences used decided upon by the investigator, materials management is not dependent on the pre–purchase of hundreds of expensive kits.

This technique does rely on certain instrumentation. A plate washer, a plate shaker, and the time–resolved fluorimeter are required. However, plate washers and shakers are common to clinical laboratories. As well, since immunoassays that use time–resolved fluorimetry have been in use in clinical laboratories for some time, these instruments may already be available and simply used for further test procedures.

This assay has proved to be sensitive and accessible to research and diagnostic laboratories. The sensitivity achieved using 50 ng/ml europium probe was comparable with that of similar studies reported in the literature and using alternate labels.

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